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Analysis of P element excision from the singed locus of Drosophila Melanogaster

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ANALYSIS OF *P* ELEMENT EXCISION FROM THE *SINGED* LOCUS OF *DROSOPHILA MELANOGASTER*. Michael C. Locker. Department of Genetics, Albert Einstein College of Medicine, Bronx, NY. (Sponsored by Lynn Cooley, Department of Genetics, Yale University School of Medicine, New Haven, CT).

P element excision from a genetic locus can be precise or imprecise. By analyzing wild-type revertant progeny of *Drosophila melanogaster* which possessed the *singed-cm* mutation (caused by insertion of a single, 628 base pair *P* element), an understanding of excision within the *singed* locus could be derived. Using cloning and subcloning techniques, as well as restriction enzyme digests and Southern blotting, the *singed* locus from the revertant flies was isolated and compared to similar DNA from wild type flies and *singed-cm* mutants. Restriction enzyme digests using EcoRI/AvaII and BalI indicated that DNA fragment lengths of revertant and wild type flies matched, while those of *singed-cm* flies differed from both revertant and wild type. Hybridization to these samples by a probe made from a full length *P* element revealed that none of the fragments in question hybridized to the probe in either the revertant or wild type flies, but hybridization did occur between probe and *singed-cm* DNA fragments. The results indicate that the *P* element that inserted into the *singed* locus in the *singed-cm* mutants has excised from the *singed* locus of the wild type revertants. Since wild type and revertant DNA fragments match closely, excision of the *P* element did not remove DNA from the *singed* locus of the revertants. Similarly, failure of *P* element probe to hybridize to revertant DNA indicates that no residual *P* element DNA remained behind after excision. Thus, excision of the *P* element from the *singed* locus is considered precise.

Analysis of *P* element excision from the *singed* locus of *Drosophila melanogaster*

A Thesis Submitted to the Yale University School of Medicine in Partial Fulfillment of the Requirements for the Degree of Doctor of Medicine

by

Michael Craig Locker

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Acknowledgements

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Introduction

Transposable genetic elements are genetic units that can insert into chromosomes, exit, and relocate (Suzuki et al., 1986). Among those transposable elements discovered in eukaryotes, perhaps the most intriguing are the *P* elements found in *Drosophila melanogaster*.

P elements are active only in germline tissues. They encompass 2907 base pairs of DNA when full-sized, but they may exist in smaller sizes due to internal deletions. In all cases, *P* elements have 31 base pair inverted terminal repeats at their ends.

When full-sized, *P* elements encode genes for transposase and repressor products. The complete elements have four open reading frames (ORFs) needed to encode transposase (Figure 1).

The history behind the discovery of *P* elements begins over 20 years ago, when Hiraizumi noted an incidence of male recombination in *Drosophila melanogaster*, a phenomenon which was normally absent in the species (Hiraizumi, 1971). In 1976, Kidwell and Kidwell reported 6 associated aberrant traits in the hybrid progeny of wild type paternal and certain maternal laboratory stock crosses: male recombination, mutation, sterility, transmission ratio distortion, chromosomal aberrations, and local increases in female recombination. They named this pattern "hybrid dysgenesis" and defined it as "a syndrome of correlated genetic traits that is spontaneously induced in hybrids between mutually interacting strains, usually in one direction only (Kidwell and Kidwell, 1976)."

Further work by Kidwell, Kidwell, and Sved the following year led to the addition of a seventh associated dysgenic trait, nondisjuction. The same team of researchers observed

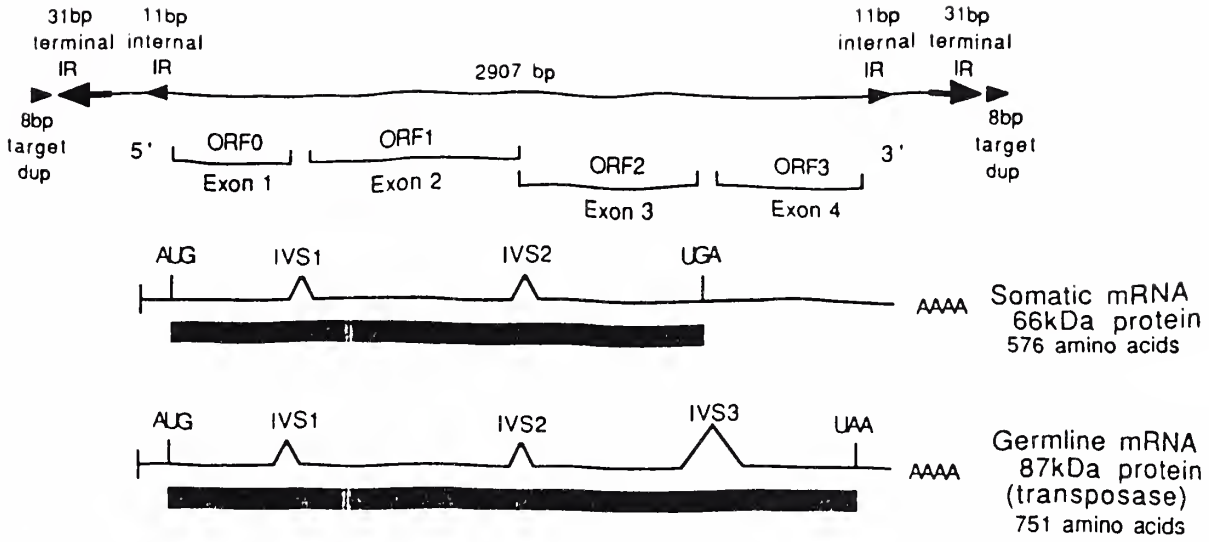


Figure 1--Structure of the 2.9 kb *P* element mRNA and proteins. Location of the four open reading frames (ORFs) or exons are shown with three introns (IVS) joining them. Terminal 31 bp and internal 11 bp inverted repeats and the direct 8 bp target site duplication are denoted by arrow heads. Translation initiation and termination codons are also shown. Black regions denote the *P* element encoded polypeptides (Rio, 1991).

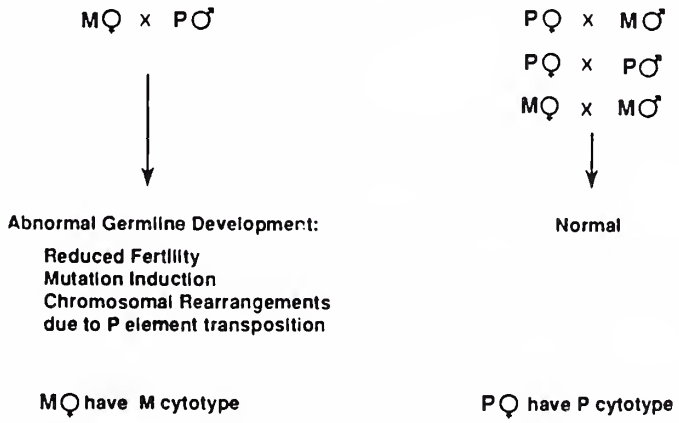


Figure 2--Hybrid dysgenesis. P strain males mated to M strain females result in symptoms of hybrid dysgenesis due to *P* element transposition, whereas reciprocal M strain males mated to P strain females or P X P or M X M progeny are normal. P strain females have P cytotype and can repress transposition by P strain sperm, whereas M strain females possess M cytotype and fail to repress transposition from P strain sperm (Rio, 1991).

that the incidence of sterility in dysgenic progeny increased with increases in ambient temperature during development.

Kidwell, Kidwell, and Sved also formulated a hypothesis for hybrid dysgenesis by proposing that "P" and "M" strains of *Drosophila* existed. The P strains consisted of the wild type stocks, so named because they were derived from the original paternal lines. The M strains were the lab stocks that represented the original maternal lines. In order to produce dysgenic progeny, M females had to be mated to P males. No other cross produced the same result (Figure 2). The basis for hybrid dysgenesis was reasoned to be controlling elements or episomes/viruses (Kidwell, Kidwell, and Sved, 1977).

Four years later, Engels expanded on this work by proposing his "P-factor hypothesis." The P "factor" responsible for hybrid dysgenesis was considered active in the M cytotype and quiescent in the P cytotype. The cytotype of the flies was controlled by the genotype. In a P-M cross, the progeny had the cytotype of the female parent. Thus, a P male X M female cross would lead to progeny with the M cytotype (P factor is active), and a M male X P female cross would lead to P cytotype progeny (P factor is quiescent) (Engels, 1981). Such a hypothesis explained the unidriectional occurrence of hybrid dysgenesis (Figure 2).

Rubin, Kidwell and Bingham (1982) lent creedence to Engels' hypothesis by renaming the dysgenic P factors "*P* elements" and by showing that most mutations seen in hybrid dysgenesis are due to these elements. The researchers looked at mutants with alterations in the *white* locus and discovered that they posessed DNA insertions of differing lengths. This DNA was homologous in sequence but heterogeneous in structure; hence all of these insertions were considered to be derivatives of *P* elements. The mutations were retained (stable) in crosses to P cytotype flies, but they had a high reversion rate to wild type when crossed to M cytotype flies. Analysis of the *revertant* flies demonstrated that the *P*

element had excised, restoring the *white* locus to its original length.

The observation that *P* element fragments of varying length could insert into the same locus of DNA led to a pair of hypotheses. First, *P* elements of varying lengths must share certain target sequences for transposase. Second, the smaller elements were derived from internal deletions and could transpose only in the presence of an intact *P* element elsewhere in the genome, which would provide the required transposase (Rubin, Kidwell, and Bingham, 1982).

In an accompanying paper, the same researchers reported that 30 to 50 copies of the element existed in a given haploid genome, and that *P* elements occurred only in P cytotype flies and in hybrids of P-M crosses. Evidence for the latter came from the observation that the *P* element inserted into the *white* locus is homologous to DNA seen in all P strains but is absent in all M strains. In addition, the *P* elements were shown to transpose in germ line tissues, not in somatic tissues, of dysgenic males (Bingham, Kidwell, and Rubin, 1982). These observations strongly supported Engels' P factor hypothesis.

The first practical usage of *P* elements in research came from Spradling and Rubin (1982), who pioneered the use of a *P* element as a basis of DNA-mediated gene transfer (vector) in *Drosophila*. They performed their experiments within the *singed* (*sn*) locus of *D. melanogaster*, and they used mutations within this locus in an assay to demonstrate the presence of *P* elements. *Singed* is a locus that, among other properties, helps determine bristle shaping on the scutellum of *Drosophila*. Spradling and Rubin used flies with the mutation *singed-weak* (sn^{w}) (slight curvature imparted to the bristle shape), crossing sn^{w} M females to P males. They noted that, in the presence of a *P* element in the paternal strains, a high rate of sn^{+} and *singed-extreme* (sn^{ext}) (more radical curvature to the bristles) progeny were produced in equal ratios (Figure 3). This experiment was repeated in various

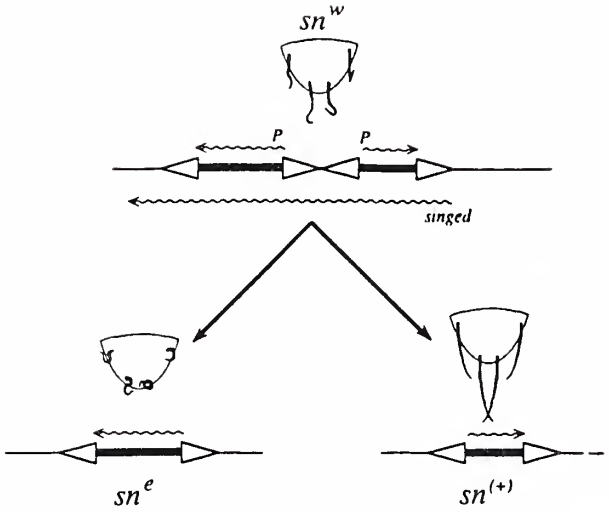


Figure 3--The *singed-weak* allele and its derivatives. The scutellum and bristles are shown for each phenotype. The sn^{w} allele mutates at high frequencies to sn^{ext} and sn^{+} in equal ratios, in the germline of dysgenic flies. Its insertion is composed of two *P* elements of lengths 1.15 and 0.95 kb, in opposite orientation. Deletions of one or the other of these elements give rise to the corresponding derivative phenotype. Wavy lines indicate transcription (Engels, 1989).

forms in other papers and can be described as the "*singed* assay."

In his 1983 review article, Engels summarized what was so far understood about *P* elements: *P* elements, which comprised 10-20% of the *Drosophila* genome, somehow become activated from a nondysgenic to a dysgenic state when the sperm of a P male fertilizes the egg of an M female. The control of this dysgenic activity involves both chromosomal and extrachromosomal factors, since P/M hybrids do not posess the same P factor activity as that seen in M/P hybrids. The M cytotype can therefore be defined as the cellular environment in which P factors are active, and the P cytotype can be defined as the cellular environment in which P factors are quiescent (Engels, 1983).

Also in 1983, the *P* element was isolated and analyzed by DNA sequencing. The full-sized element was 2907 base pairs in length, had multiple areas of highly conserved sequences, was bounded by 31 base pair "inverse terminal repeats", and had at least 3 open reading frames. The smaller *P* elements appeared to have arisen from internal deletions of full-length *P* elements. *P* elements were classified as a new type of transposon that differed from others in its structure and in its ability to be experimentally manipulated in rate of transposition. It was equated to the P factors proposed by Engels (O'Hare and Rubin, 1983).

In 1984, 4 areas of the *P* element were identified as essential for its hypermutable behavior (Karess and Rubin, 1984). With the isolation and sequencing of *P* elements, and their recognition as a new class of transposable genetic element responsible for hybrid dysgenesis, the ground was broken for a better understanding of the mechanism behind hybrid dysgenesis, the basis of P and M cytotype, models of *P* element transposition, and a stronger grasp of *P* element germline specificity.

Although a definition of hybrid dysgenesis (Kidwell and Kidwell, 1976) and its 7 associated traits (Kidwell and

Kidwell, 1976; Kidwell, Kidwell, and Sved, 1977) had been recognized, a better understanding of the effects of *P* elements on hybrid dysgenesis emerged with further research. Gonadal dysgenic sterility, the presence of rudimentary gonads in either sex, without any effect on somatic tissues, sometimes occurred unilaterally (Engels and Preston, 1979), and its frequency was temperature dependent (Kidwell and Novy, 1979). An early loss of germ cells evolved as one explanation for this trait (Engels, 1983).

The numerous mutations associated with hybrid dysgenesis were classified more easily after a better knowledge of *P* elements developed. Dysgenic mutations were found to occur from *P* element insertion, precise/imprecise excision, or chromosomal rearrangement with at least one breakpoint at the site of a *P* element (Engels, 1983). The ability of *singed-weak* to undergo hypermutability and produce *singed-extreme* and wild type *revertants* in equal ratios was singled out as a special case of mutation (Engels, 1983).

In addition, the chromosomal aberrations associated with dysgenesis were observed to be caused by inversions, translocations, and transpositions (Engels, 1983). Furthermore, the dysgenic traits seemed to possess certain common characteristics: all were suppressed by P cytotype; all were limited to the germline; as a whole, the dysgenic traits demonstrated that the activity of *P* elements was not limited to any single developmental stage; and, excepting gonadal dysgenic sterility, all the traits had only slight variation in frequency with temperature (Engels, 1983).

As understanding of *P* elements increased, models to explain the P and M cytotypes appeared. O'Hare and Rubin (1983) proposed that the *P* element produces a regulator product in eggs, absent in sperm, which has a negative effect on the product transposase and a positive feedback on its own production.

In 1989, Robertson and Engels conducted a series of experiments to support the theory that a *P* element-encoded

repressor of transposition and excision existed. Robertson and Engels introduced two sensitive assays as part of their work: the first assay involved an insertion of a *P* element into *singed* which would cause the female's egg phenotype to be dependent upon cytotype; the second assay employed the standard *singed* assay to look for cytotype-dependent bristle morphology in hypermutant sons, and cytotype-dependent egg morphology in hypermutant daughters.

It was observed that egg morphology became more mutated in daughters with P cytotype, indicating that the *P* element-encoded product acts in *trans* with the *P* element insertions in the *singed* locus, altering the expression of the mutant *singed* gene--i.e., the presence of a *P* element-encoded repressor product could be determined by egg morphology.

After establishing the validity of these assays, the same workers isolated modified *P* elements that still (by assay) retained the ability to produce repressor despite having internal modifications. These same elements, when present in the genome, repressed the hypermutability of *singed-weak* to *singed-extreme* and wild type, as well as repressing gonadal-dysgenic sterility and somatic mosaicism in other relevant assays. All of these assays were dependent on an M cytotype female cross for positive results. Alteration of the results was produced by crossing to females carrying the modified *P* elements that retained repressor function. The result supported the hypothesis that P cytotype is derived from *P* element-encoded repressor function (Robertson and Engels, 1989).

Along with greater understanding of *P* element structure came the development of models to explain element transposition. Many of these models are derived from the recombinational models proposed for fungi, which include those of Holliday, Meselson-Radding, and double strand break repair. Holliday first hypothesized that heteroduplex DNA may be generated during the breakage and joining of chromosomes, as a result of pairing of nicked sister

chromatid strands. The ensuing Holliday junction could be cleaved two different ways, causing different types of chromosomal segregation (Figures 4 and 5) (Orr-Weaver and Szostack, 1985).

The Meselson-Radding model elaborates upon that of Holliday by attempting to explain asymmetric heteroduplex DNA. Only one chromatid is proposed to be nicked, and it then invades the other chromatid to form a D-loop (Figure 6). After branch migration or isomerization, a Holliday junction results, and the Holliday model then follows as a second, symmetric phase of recombination (Orr-Weaver and Szostak, 1985).

Double strand break repair is the most complex model of recombination. Again, it elaborates on the preceding theories. As in the Meselson-Radding model, a D-loop is proposed to result from an invading nicked sister chromatid. However, gap repair occurs in such a fashion that 2 regions of heteroduplex DNA are formed, one junction like that proposed in the Meselson-Radding model, the other like a Holliday junction. Depending upon where the strands are cut, symmetric heteroduplex DNA, asymmetric heteroduplex DNA, or gene conversion can result (Figures 7, 8, and 9) (Orr-Weaver and Szostak, 1985).

O'Hare and Rubin (1983) first recognized that the mechanism by which *P* elements transpose is unique. Using mutations in the *white* locus, they noted that 8 base pairs of DNA are duplicated in direct orientation upon insertion of a *P* element, due to a staggered cut in the target site made by the transposase product (Figure 1). The same 8 duplicated bases were removed along with the complete *P* element during excision, also as the result of a staggered cut. By isolating extremely small elements, they concluded that only the first 138 and last 216 base pairs were required for insertion or excision, and they proposed that the *P* elements themselves coded for transposase and its regulator.

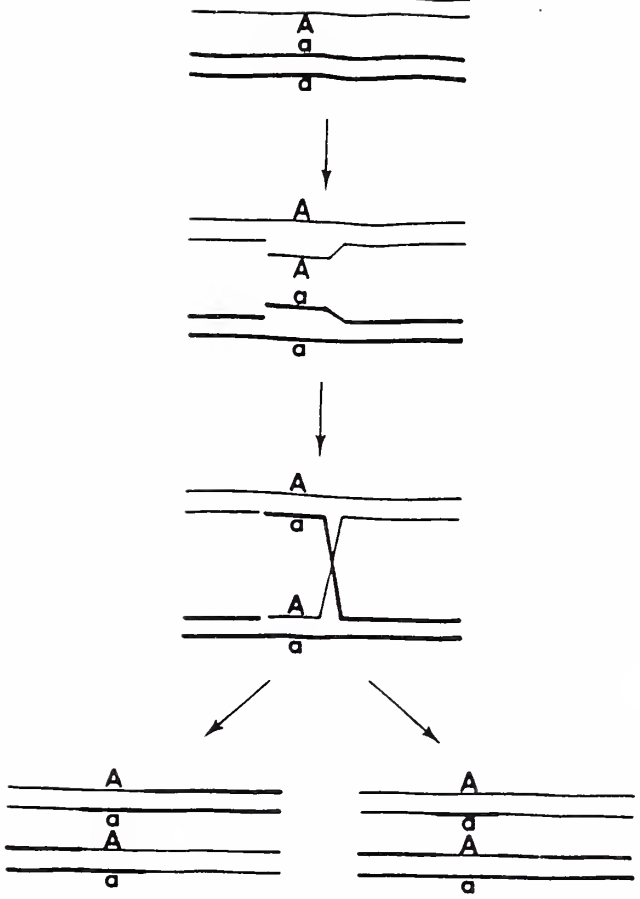


Figure 4--Holliday model for recombination. Strands of the same polarity are nicked at homologous sites and are then exchanged to produce heteroduplex DNA. The crossed strand, or Holliday junction, can be resolved either with or without exchange of flanking markers. If the symmetric heteroduplex produced by strand exchange is not repaired, an aberrant 4A:4a segregation results (Orr-Weaver and Szostak, 1985).

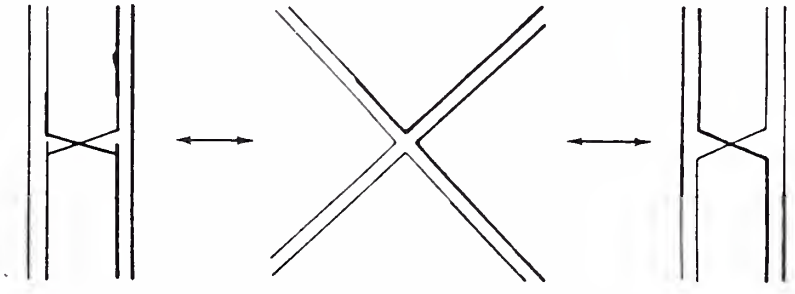


Figure 5--Isomerization of a Holliday junction. The junction can isomerize through a symmetrical intermediate without bond breakage. Thus, resolution can occur by cutting either the originally crossed strands or the nonncrossed strands (Orr-Weaver and Szostak, 1985).

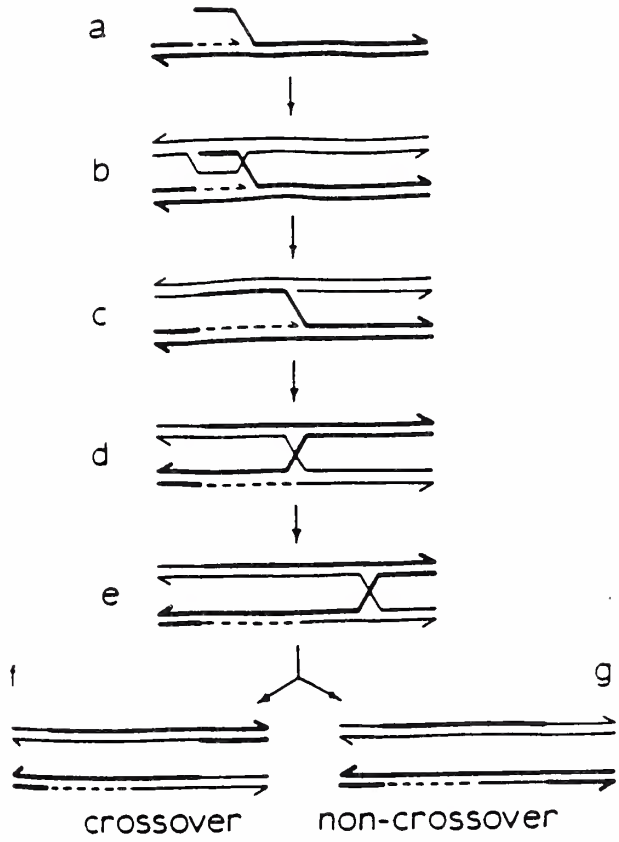


Figure 6--Meselson-Radding model. (a) Recombination is initiated by a single-strand nick which serves as a primer for DNA repair synthesis. This displaces a single strand which can then pair with a homologous region on the other chromatid (b). The resulting D-loop is degraded, and the asymmetric heteroduplex DNA is enlarged by DNA synthesis on the donor chromatid coupled with degradation on the recipient duplex (c). Branch migration and ligation of the nicks produces a Holliday junction which can be isomerized (d). Symmetric heteroduplex DNA can be formed by branch migration of the Holliday junction (e). Resolution can yield either the crossover (f) or the non-crossover (g) configuration (Orr-Weaver and Szostak, 1985).

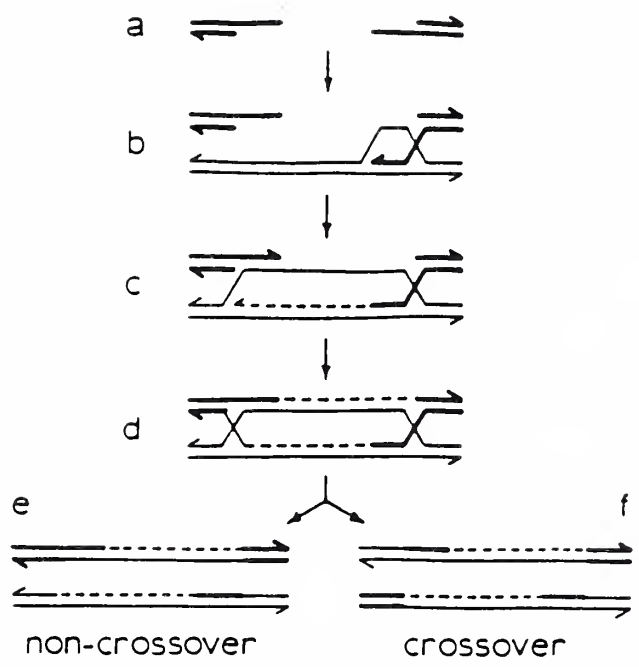


Figure 7--Double-strand break repair model. (a) A double-strand cut is made in one duplex, and a gap flanked by 3′ single strands is formed by the action of exonucleases. (b) One 3′ end invades a homologous duplex, displacing a D-loop. (c) The D-loop is enlarged by repair synthesis until the other 3′ end can anneal to complementary single-stranded sequences. (d) Repair synthesis from the second 3′ end completes the process of gap repair, and branch migration results in the formation of two Holliday junctions. Resolution of the junctions by cutting either the inner or outer strands leads to two possible non-crossover (e) and two possible crossover (f) configurations. In the figure, the right hand junction was cut at the inner, crossed strands (Orr-Weaver and Szostak, 1985).

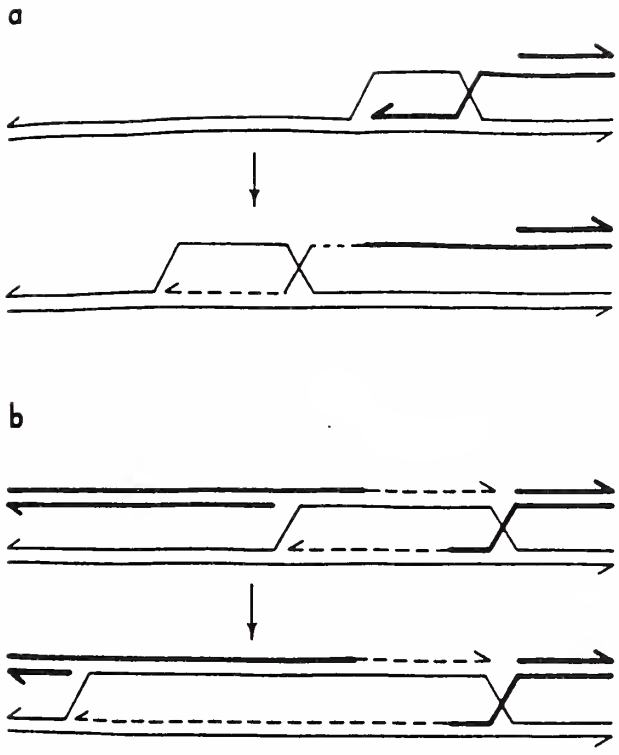


Figure 8--Formation of asymmetric heteroduplex DNA on only one chromatid. Initiation as shown creates heteroduplex DNA on the right-hand side of the gap by strand invasion and on the left hand side of the gap by strand annealing. To avoid the formation of aberrant 5:3 product, all heteroduplex DNA must be confined to one chromatid, and one Holliday junction must be resolved in a defined way. The right-hand heteroduplex DNA may be removed by single-stranded branch migration as the first round of repair synthesis is in progress (a). Alternatively, the right-hand region of heteroduplex DNA may be quite short, and the left-hand region may be extended enzymatically, so that essentially all observed postmeiotic segregation derives from the left-hand heteroduplex (b) (Orr-Weaver and Szostak, 1985).

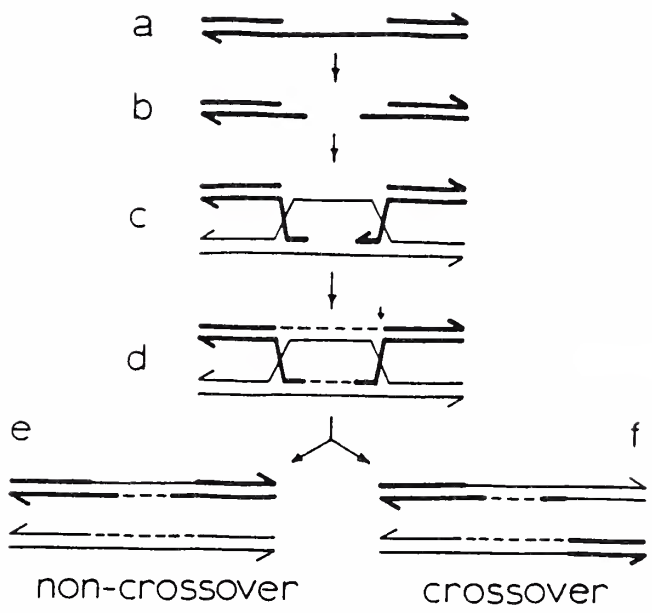


Figure 9--Initiation from overhanging 5′ and 3′ ends. Initiation occurs by the formation of a single-strand gap (a), which is then cleaved by nucleases to produce overhanging 5′ and 3′ ends (b). These ends invade the homologous duplex (c), and the double-strand gap is repaired by two rounds of single-stranded repair synthesis (d). This initiation model confines all heteroduplex DNA to the donor chromatid; to avoid the generation of aberrant 5:3 product, one Holliday junction must be resolved by cutting the outer strands. After the completion of the second round of repair synthesis, one of the outer strands of the right-hand Holliday junction is already nicked. This nick could bias the resolution of the Holliday junction towards cutting of the outer strands. Resolution of the left-hand junction can then result in a non-crossover (e) or crossover (f) configuration (Orr-Weaver and Szostak, 1985).

The following year, Engels used the hypermutability of *singed-weak* in a set of experiments designed to understand transpositional activity. He noted that sn^{w} flies with M cytotype lacking hypermutability gained it back when *P* elements were introduced onto autosomes. The result indicated that the *P* element at the *singed* locus must have been deficient in transposase production, and that such defective elements need transposase provided by other elements (acting in *trans*) in order to transpose. His molecular analysis also indicated that sn^{w} was the result of the insertion of a two *P* elements, while sn^{ext} resulted from the loss of one element, and wild-type *revertants* resulted from the loss of the other element (Figure 3) (Engels, 1984).

Hawley et al. (1988) used a variant of the *singed* assay to propose a new model of transposition. They examined the *singed-cm* mutation, which, like sn^{w}, resulted in a moderate curling of bristle shape, caused by the insertion of a 0.6 kilobase *P* element. This mutation is also hypermutable to *revertant* and *extreme* phenotypes (Figure 10), with the *revertant* having no *P* element present, and the *extreme* phenotype possessing a second 0.6 kb element in inverted alignment with the first.

To explain their observations, Hawley et al. proposed a model of intracistronic conservative transposition, which is as follows: the excision of the *P* element from one chromatid and its insertion into the sister chromatid produces the *extreme* phenotype; the second *P* element is assumed to be a copy, and excision is precise (Figure 11). Such a model explains the equal frequencies of *revertant* and *extreme* phenotypes seen in hypermutant progeny. It does not account for the frequent imprecise excisions that were observed (Hawley, 1988).

A second model of replicative intracistronic transposition, given less favor, contended that excision and duplication of the *P* element involved in hypermutation to *extreme* phenotype are unrelated events. Duplication is thus

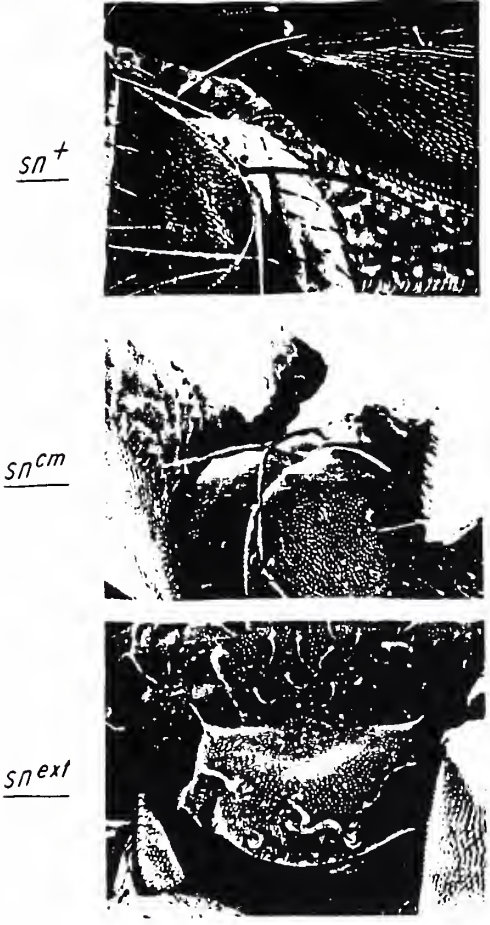


Figure 10--Scanning electron microscopy photographs of the posterior scutellar bristles of *sn*$^{+}$, *sn*^{cm}, and *sn*^{ext} males (Hawley et al., 1988).

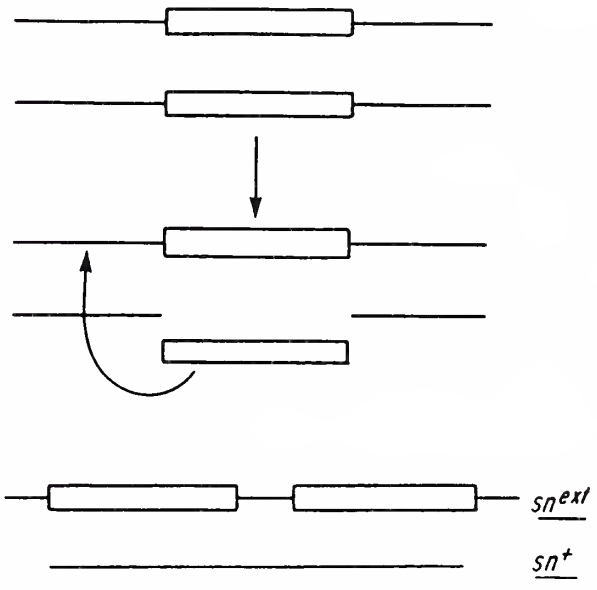


Figure 11--Model of intracistronic conservative transposition. *sn*^{ext} and *sn*$^{+}$ result from the excision of one *P* element from one chromatid and its resulting insertion onto the other chromatid (Hawley et al., 1988).

taken to be reflective of the tendency of *P* elements to transpose to sites near the previous insertion of an element, or, some unknown factor retards other types of *P* element from transposing to the *singed* locus. This model cannot explain how *revertants* are generated or why equal ratios of *revertant* and *extreme* phenotype flies are produced (Hawley et al., 1988).

A third model, that of interlocus transposition, postulated that another 628 base pair *P* element inserted into *singed* from another locus, thereby causing the *extreme* phenotype. The drawback of this model is that few 628 base pair elements exist in the genome, and no other studies had indicated that certain sized elements selectively migrate to certain genomic hotspots (Hawley et al., 1988).

Perhaps the strongest contribution to the understanding of *P* element transposition comes from the work of Engels et al. (1990), who proposed a "cut and paste" mechanism of transposition, followed by double-strand gap repair. They focused on the *white* (*w*) locus, using an assay in which progeny of mutant w^{hd} flies hypermutated to w^{+} *revertants*. When w^{hd} was hemizygous, hypermutation to wild type was low. Reversion rate increased in heterozygous flies with alleles having non-overlapping regions. By following genetic markers around the w^{hd} gene (flanking markers), the researchers observed that *revertant* flies posessed the flanking marker configuration, indicating that they were derived from w^{hd}. Analysis of *revertant* DNA showed that excision was precise. They concluded that reversion was dependent upon the presence of a wild type homolog.

Conversely, when w^{hd} was paired with a balancer chromosome, a decrease in rate of reversion resulted. Similarly, when w^{hd} was paired with a wild-type allele in an ectopic location, a reversion rate that was slightly lower than that seen for non-ectopic pairing ensued. In light of this additional work, the dependence of reversion rate upon homologs suggested that a transfer of genetic information

occurred between the disrupted locus and the wild-type homolog. Similar results were shown at the *singed* locus (Engels et al., 1990).

However, *P* element insertion was observed to be independent of the presence of a homolog, and it occurred much more frequently than precise excision. This excess number of transpositions when compared to precise excisions ruled out any nonreplicative transposition models of excision (Engels et al., 1990).

In forming their new model, Engels et al. summarized the following pertinent information: internal excision requires *P* element transposase and is blocked by *P* element repressor; excisional breakpoints are variable, but there is a preference for short direct duplications of 3 or more base pairs; short duplications or triplications are sometimes generated at the breakpoints, implying that DNA synthesis is involved in excision; and, all internal excisions are at pre-existing *P* element sites, implying that if internal deletions are coupled to transposition events, partial loss must occur at the donor site, not at the recipient site (Engels et al., 1990).

Taking all this information into account, the researchers proposed (Figure 12) that the excision of a *P* element from one homolog leaves a double-strand gap, which may be widened. The excised element then could move elsewhere within the genome. One or both of the broken ends of the gap then must invade a homologous duplex to initiate double-strand gap repair; the invaded duplex serves as a template for DNA synthesis. This template may be either the sister chromatid (the usual case) or the homologous chromosome (rarer). If the homologous chromosome is the wild type, precise excision results. The frequent internal deletions observed must be due to incomplete repair (Engels et al., 1990).

This model can thus account for male recombination, one of the dysgenic traits, and several predictions result:

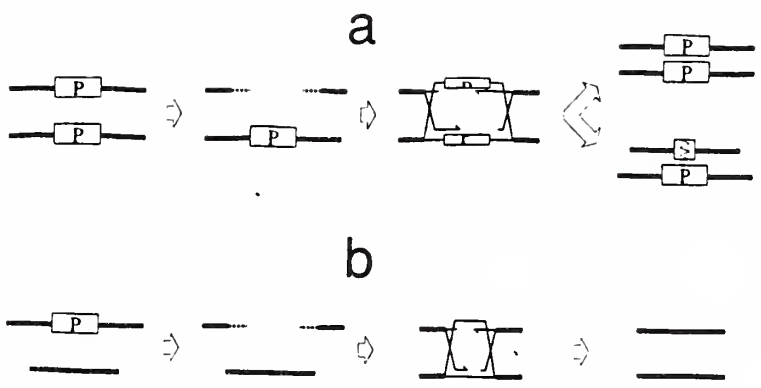


Figure 12--Double-strand gap repair model of *P* element transposition and excision. The top duplex of each figure represents the site of a *P* element undergoing transposition, and the bottom duplex is either a sister chromatid or homologous chromosome to be used as the template for gap repair. In the first step, the *P* element is excised to be reinserted elsewhere. The dotted lines represent possible exonuclease activity. Such activity is required if the excision leaves behind both copies of the host sequence duplication, but it might occur in any case. The second step represents double-strand gap repair in which genetic information is copied from the template duplex to the site of the excision. (a) When the template is a sister chromatid, a copy of the original *P* element can be generated (top). If the gap repair process is interrupted (bottom), the result moght be an internally-deleted *P* element. (b) when the template is a homologous chromosome with a wild-type sequence at the insertion site, then gap repair regenerates the wild type sequence. The result is a reversion (Engels et al., 1990).

first, that information from both strands of the donor template will be incorporated into the recipient; second, that extrachromosomal copies of the *P* element could be recovered as transposition intermediates; and third, that only those mutations that affect gene conversion will affect *P* element loss. Interestingly, the model can be viewed both as a form of nonreplicative transposition, since both donor strands migrate, and as a replicative model, since there is a transposition to a new insertion site with a retaining of the original site (Engels et al., 1990).

Thus far, one set of researchers has examined the cut and paste model and found evidence to support it. Gloor et al. (1991) demonstrated that sequence differences in the flanking regions of the template DNA can be copied to the site of *P* element excision (Figure 13).

As the basics of *P* element structure were ironed out, a further understanding of germline specificity developed. Germline specificity was found to be controlled at the level of mRNA splicing, not at transcription (Laski, Rio, and Rubin, 1986). This discovery resulted from looking at different *P* element mutations and determining which were able to create transposase, thus allowing sn^{w} flies to create the hypermutant progeny sn^{+} and sn^{ext}. Only *P* elements with an intact splice between open reading frames 2 and 3 possessed this ability.

The same researchers discovered the existence of a third intron and a fourth open reading frame. The third intron's removal was required for transposase production, while all 4 ORFs were required for a complete transposase product. The basis of germline specificity was reasoned to be the removal of the third intron in the germline tissues only, and the lack of somatic *P* element activity suggested that regulation was not at the level of transcription. Furthermore, the *P* element promoter could not be the factor controlling specificity, since it is active in the somatic tissues (Laski, Rio, and Rubin, 1986).

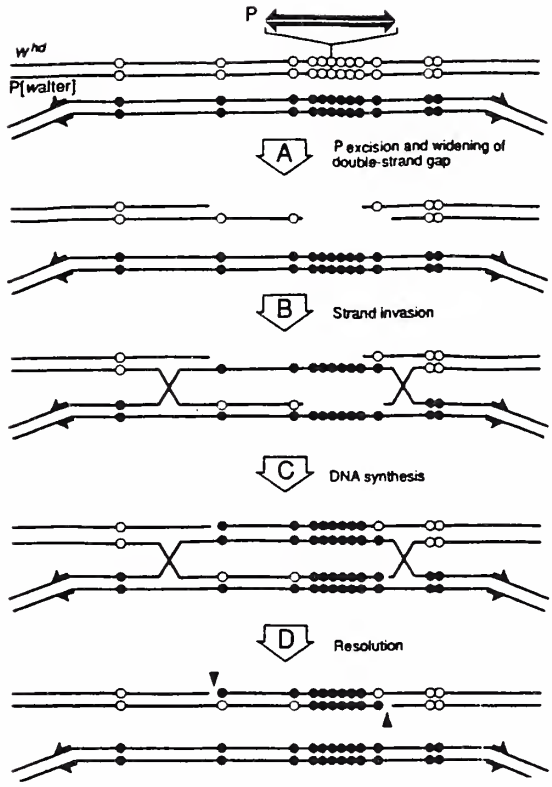


Figure 13--Gloor et al.'s experiment testing the double-strand gap repair model. The double-strand break is within the *P* element insertion allele w^{hd}, while the template is an ectopic *white* gene carried on *P[walter]*. The filled and open circles indicate differing marker sequences. (A) Formation of double-strand gap. (B) Broken ends and *P[walter]* associate, and strand invasion begins. (C) Polymerization creates two Holliday junctions. (D) Resolution of Holliday junctions., creating a central region of converted sites flanked by heteroduplex regions. Single-strand nicks (arrows) are ligated (Gloor et al., 1991).

Three years later, Laski and Rubin (1989) showed that the same intron, that between ORFs 2 and 3, conferred germline specificity when placed in the context of another gene. They further speculated that certain "factors" interact with *P* element pre-mRNA at specific sequences. These factors would either be germline specific and required for splicing, or they would be limited to the soma and inhibit splicing. Further work showed that the product of the *P* element was an 87 kiloDalton polypeptide in germ cells and a shortened 66 kDa polypeptide in the soma (Figures 14 and 15) (Robertson and Engels, 1989).

As has been shown, the *singed* locus has been at the center of much of the research focused on *P* elements. This mutation, first occurring spontaneously in 1922, produces shortened and twisted bristles on the scutellum of *Drosophila* (Figure 16). The hairs are wavy, and the females are sterile. Mapping has put the locus on chromosome 1, area 21.0 (Figure 17) (Lindsley and Grell, 1968).

The *singed-weak*, *singed-extreme*, and wild type *revertants* that occurred as part of Spradling and Rubin's original assay (1982) were analyzed further in 1988 using molecular techniques (Roiha, Rubin, and O'Hare). The *singed-weak* mutation was shown to be caused by a 2.1 kilobase insert consisting of 2 P elements--one 1.15 kb, the other 0.95 kb, in inverted orientation. Each of the hypermuatant progeny possessed only one of these inserts--sn^{ext} having the 1.15 kb element, and the *revertants* having the 0.95 kb element.

Furthermore, *singed* was shown to be a hotspot for these transposons for reasons other than size or sequence, since insertions occurred within a 700 base pair region, and since the most frequently used site of insertion was not the best 8 base pair sequence match with the element (Roiha, Rubin, and O'Hare, 1988). The high reversion rate found with sn^{w} was theorized to come from the association of 2 *P* elements at the locus (Roiha, Rubin, and O'Hare, 1988).

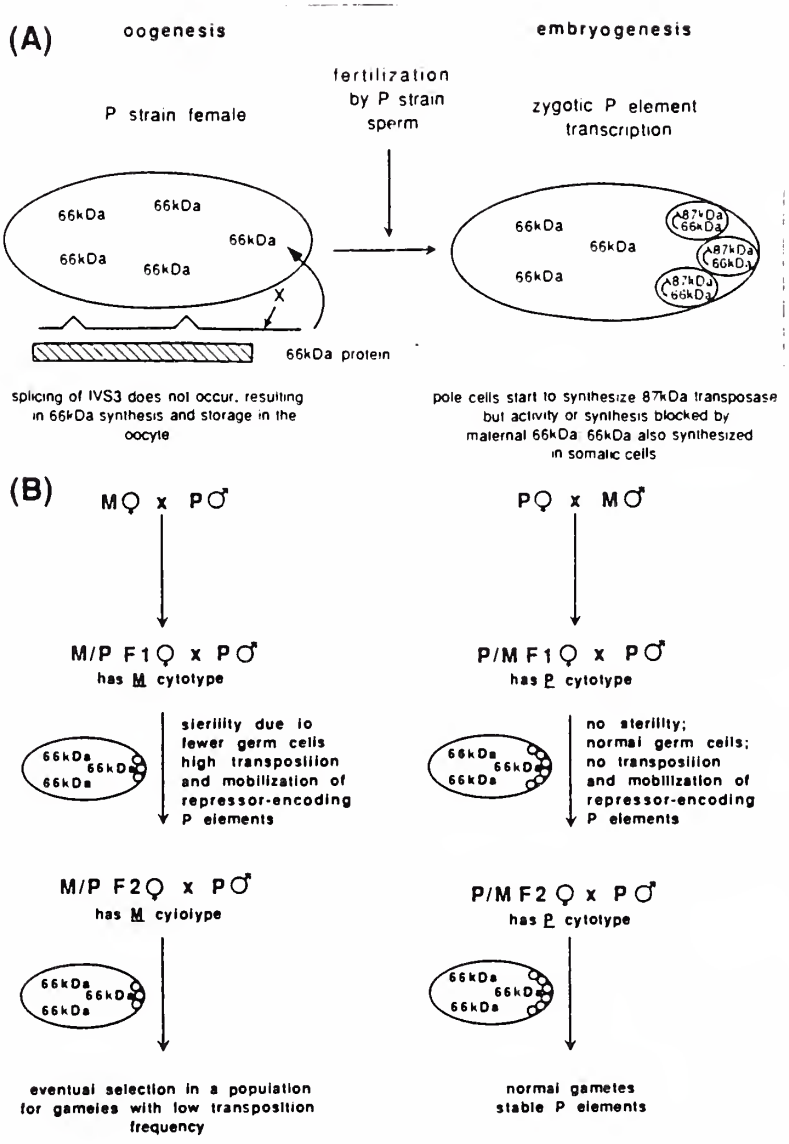

(A)
oogenesis
embryogenesis
fertilization by P strain sperm
P strain female
zygotic P element transcription
66kDa
66kDa
66kDa
66kDa
66kDa
X
66kDa protein
66kDa
66kDa
66kDa
87kDa 66kDa
87kDa 66kDa
87kDa 66kDa
splicing of IVS3 does not occur, resulting in 66kDa synthesis and storage in the oocyte
pole cells start to synthesize 87kDa transposase but activity or synthesis blocked by maternal 66kDa. 66kDa also synthesized in somatic cells
(B)
M♀ x P♂
P♀ x M♂
M/P F1♀ x P♂
has M cytotype
P/M F1♀ x P♂
has P cytotype
66kDa
66kDa
66kDa
sterility due to fewer germ cells high transposition and mobilization of repressor-encoding P elements
66kDa
66kDa
66kDa
no sterility; normal germ cells; no transposition and mobilization of repressor-encoding P elements
M/P F2♀ x P♂
has M cytotype
P/M F2♀ x P♂
has P cytotype
66kDa
66kDa
66kDa
66kDa
66kDa
66kDa
eventual selection in a population for gametes with low transposition frequency
normal gametes stable P elements

Figure 14--Model for the role of maternal 66 kDa protein in repression of transposition in P strains (P cytotype). (A) The 66 kDa protein is made during oogenesis by failure of the ORF2-3 to splice (X) in the nurse cells, resulting in deposition of the protein in the oocyte. The 66 kDa protein then represses synthesis of the 87 kDa transposase. (B) Maternal inheritance of P cytotype in M/P and P/M hybrids. P cytotype is inherited from P strain females when P/M hybrids are backcrossed to P strain males (right), and repressor-encoding elements remain in positions that allow expression during oogenesis, resulting in inheritance of P cytotype. However, M/P hybrids, which should be genotypically identical, are not, because high frequency of transposition mobilizes the *P* elements. This results in sterility, fewer germ cells, and movement of maternal oogenesis-expressing repressor-encoding elements, preventing expression of repressor during oogenesis. Therefore, M/P hybrids exhibit M cytotype (Rio, 1990).

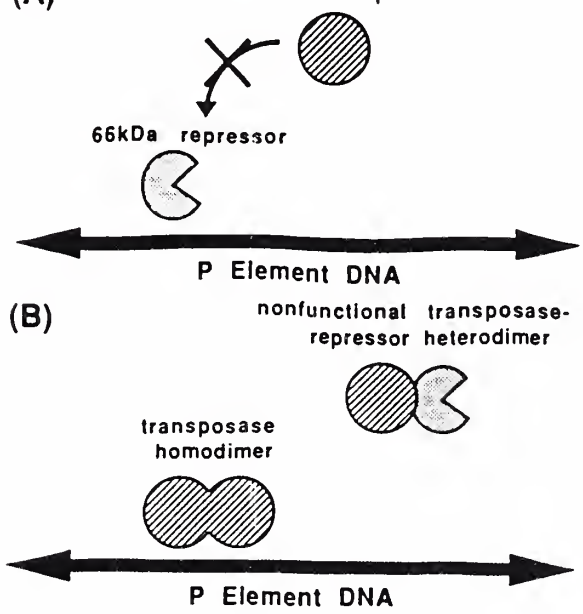
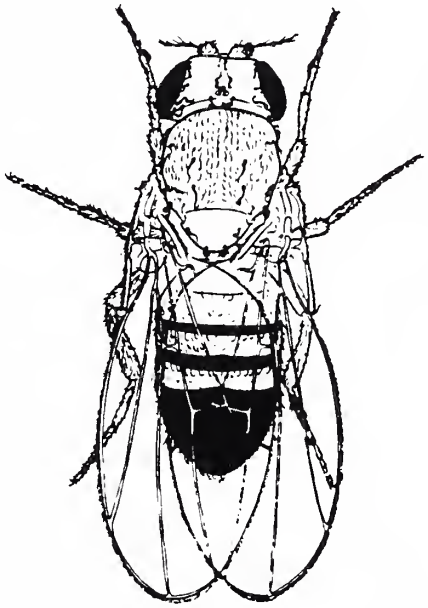


Figure 15--Models for the repression of transposition by truncated transposase proteins. (A) DNA binding--may be on or near transposase-binding sites, thereby blocking transposase action (arrow with X). (B) Protein-protein interactions--transposase may bind to DNA as a dimer. The dimer may be inactivated by interaction with the 66 kDa protein (Rio, 1990).



sn: singed

Figure 16--*singed* phenotype (Lindsley and Grell, 1968).

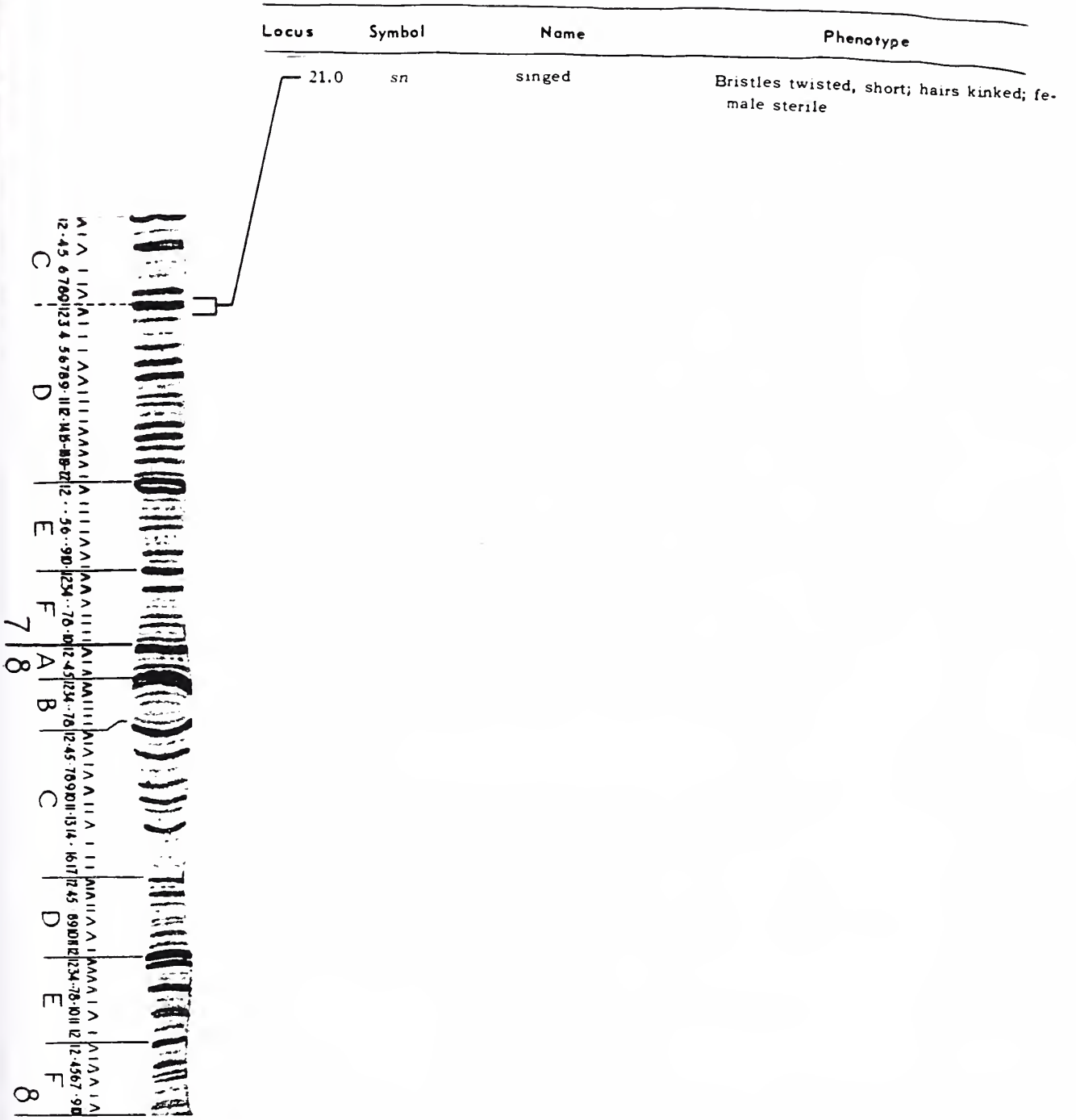


Figure 17--Location of *singed* on chromosome 1 (Lindsley and Grell, 1968).

The mutation *singed-cm* is 0.6 kilobases longer at its 5′ end than that of the wild type (Hawley et al., 1988). Isolation of the *P* element involved put its exact length at 628 base pairs, due to an internal deletion from 139-2416 (O'Hare and Rubin, 1983). This mutant's *extreme* progeny had a duplication and inversion of a similar 628 bp element, while the revertant's DNA was indistinguishable from that of the wild type.

An interesting recent discovery regarding *singed* is that the mutation affects the female germline in addition to affecting bristle shape (Paterson and O'Hare, 1991). Based on the observation that *singed* mutants are often sterile, RNA from the locus was examined and found to be of 3 distinct sizes: 3.0 kb length RNA was observed in embryos, 3.3 kb was seen in females only, and 3.6 kb was found universally. All these RNAs are transcribed distally to proximally during metamorphosis and oogenesis. They transcribe the same product and differ only at the 3′ untranslated end.

More importantly, *singed* mutants seem to have decreased bundles of microfibrils (Overton, 1967). Thus, *singed* may have a role in reorganization or redistribution of microfilaments.

The work contained within this thesis involves an analysis of the wild type *revertants* isolated from *singed-cm* parents in the Hawley laboratory. Investigation using molecular techniques focused on whether excision of the 628 base pair *P* element was precise or imprecise.

Materials and Methods

(The following procedures are from Maniatis et al., 1982, except as noted)

Construction of genomic libraries. Genomic DNA from 3 different strains of *singed revertant Drosophila melanogaster* was obtained from the Hawley laboratory. The

three different DNA samples were labelled *sn*^{rev} *645-15*, *sn*^{rev} *645-35*, and *sn*^{rev} *645-60*.

These samples, along with lambda *gt10* DNA, were subjected to an EcoRI restriction enzyme digest. 10 µl of the digestion was run on a 0.6% agarose gel to confirm a thorough digestion. The samples were then extracted two times with phenol, phenol/chloroform (1:1), and one time with chloroform. NaCl was added to a final concentration of 0.3M, and the DNA was precipitated with two volumes of ethanol at -70° C for 30 minutes. After centrifugation at 8 krpm for 15 minutes at 4° C, the pellet was washed with 0.5 ml 70% EtOH and recentrifuged 5 minutes at 4°. The pellet was resuspended in 6 µl TE buffer.

The *revertant* DNA fragments were ligated to the lambda DNA using T4 ligase and ligase buffer overnight at 37°.

Titering genomic libraries. C600*Hfl*$^{+}$ bacteria were innoculated in LB broth and grown overnight. The ligated DNA was added to 100 µl of cells in doses of 5 µl undiluted, 1 µl undiluted, 10 X 10^{-2} µl, 1 X 10^{-2} µl, and 10 X 10^{-4} µl and vortexed to mix. The cells and DNA were incubated at 37° for 20 minutes.

Afterward, 3 ml of melted 0.7% agarose was added to the incubating tubes, then vortexed, and the tubes were poured onto LB plates. These were closed and inverted, then incubated at 37° overnight.

Plating genomic libraries. After examining the density of growth on the incubated plates, the titer was determined, and the appropriate amount of phage was combined with cells and plated out, as above.

Screening libraries for *singed* DNA. A dry NYTRAN filter sheet was placed onto the library plates for 3 minutes. After removing this filter, a second sheet was placed on the plates for 4 minutes. Both plates and filters were marked with ink for future realignment. The filters were removed with a forceps and placed into 0.1N NaOH, 1.5M NaCl for 1 minute, then into 0.2M Tris-HCl (pH 7.5), 2 X SSC

for 1 minute. The filters were allowed to air dry, then were baked for 1 hour at 80°.

The same filters were then prehybridized in 50% formamide, 5 X SSC, 5 X Denhart's solution, 500 µg/ml salmon sperm, and 0.1% SDS at 42° for 1 hour, then hybridized to probe *psn9* (a wild type copy of the 5' end of *singed*) overnight.

The *psn9* probe was made by combining the appropriate DNA with water, boiling 5 minutes, then chilling on ice for 10 minutes. To this solution was added BSA and labelling buffer, ^{32}PαdCTP, and Klenow DNA polymerase. After sitting at room temperature 3 hours, 0.25M EDTA is added. The solution is boiled 2 minutes, then cooled 10 minutes.

After hybridization, the filters were washed with 2 X SSC and 0.1% SDS, and allowed to dry. The filters were then exposed to film overnight (autoradiogram).

Picking plaques. Autoradiograms of the hybridized filters were developed and aligned to the proper plates such that positive plaques could be picked. The appropriate plaques were stabbed with the sterile end of a Pasteur pipette and resuspended in 1 ml of phage buffer and replated as above for a secondary screening. The resulting plates were again lifted with NYTRAN and hybridized with *psn9* probe. The appropriate colonies were again identified, picked, and resuspended and replated for a tertiary screening.

Preparation of phage DNA. After the tertiary screening, a single pure plaque of appropriate DNA was obtained and titered so that replating yielded 50-100 plaques per plate. After refrigerating the plates, 10-30 plaques were picked and suspended in 200 µl of phage buffer. 200 additional µl were added after vortexing. This suspension was put on ice 2 hours with frequent vortexing.

Afterwards, 8 µl of *LE392* cells were added to the suspension from an overnight culture, and the phage/cell mixture was incubated for 20 minutes at 37°. 15 ml of NZY broth was added, and the mixture was shaken overnight at 37°.

The following day, 5 drops of chloroform were added, and the solution sat for 30 minutes. After centrifugation at 8 krpm for 10 minutes at 4°, the supernatant was poured off into a sterile tube, to which was added 20 µl RNase and 15 µl DNase. This was incubated for 30 minutes at 37°. An equal volume of 20% polyethelene glycol and 2M NaCl in phage buffer solution was added, and the mixture was incubated 1 hour at 0°.

After centrifugation at 10 krpm for 20 minutes at 4°, the supernatant was aspirated and the tube was inverted. The pellet was resuspended in 0.5 ml phage buffer, vortexed, and transferred to an Eppendorf tube. This was centrifuged at 8 krpm for 4 minutes at 4°, and the supernatant was transferred to a fresh tube. To the fresh tube was added 5 µl 10% SDS and 5 µl 0.5M EDTA (pH 8.0), and the solution was incubated 15 minutes at 68°.

The solution was extraced with equal volumes of phenol, phenol/chloroform, and chloroform, and an equal volume of isopropanol was added to the final aqueous phase. This sat 30 minutes at -70°. After thawing, it was centrifued at 8 krpm for 15 minutes at 4°. The supernatant was dumped, and the pellet was washed with 500 µl 70% ethanol. This mixture was centirfuged 5 minutes at 8 krpm at 4°.

The resulting pellet was dried and resuspended in 100 µl TE buffer. A test sample of the phage DNA was analyzed by an EcoRI restriction enzyme digest to confirm the presence of the appropriate DNA.

Subcloning of phage DNA into *pT7-1* plasmid. 30 µl phage DNA and 2 µg *pT7-1* plasmid DNA were digested with EcoRI and SalI. After extraction with phenol, phenol/chloroform (equal volumes), and chloroform, the DNAs were combined, then precipitated with 2 volumes of ethanol for 30 minutes at -70°. The tube was centrifuged at 8 krpm for 15 minutes at room temperature, then washed with 70% EtOH. Afterwards, the supernatant was removed, and the pellet was dried and resuspended in 10 µl of TE buffer.

To 8 µl of sample DNA was added 1 µl each of ligase and ligase buffer, and the ligation occurred overnight at room temperature. To verify the ligation, 2 µl of DNA were removed and run on an agarose minigel.

Transforming cells. Single colonies of C600*Hfl*$^+$ cells were innoculated into LB broth and incubated at 37° overnight. 10 µl of this culture was suspended in 100 µl of LB and incubated overnight. The cells were collected by centrifugation at 8 krpm for 10 minutes at 4°. The pellet was resuspended in 10mM NaCl at 4°, then centrifued again at 8 krpm for 10 minutes at 4°. The pellet was resuspended in 30mM $CaCl_2$ at 4°; after incubation of 30 minutes at 4°, centrifugation was repeated as above, and the pellet was resuspended in 1.5mM $CaCl_2$ at 4°.

5 µl of the ligated sample were added to 300 µl of the competent cells. The mixture was incubated for 60 minutes at 4°, then heat shocked at 42° for 2 minutes. The heat shocked cells were added to 2.7 ml LB and incubated for 60 minutes at 37°. Using 10-fold dilutional titers, the cells were plated on LB plates.

Plasmid minipreps by alkaline lysis. Colonies from the LB plates of transformed cells were picked using sterile Pasteur pipettes, added to 5 ml LB broth, and incubated overnight. The following day, 1.5 ml of the culture was poured into Eppendorf tubes, centrifuged for 1 minute at room temperature, and aspirated to leave a bacterial pellet. The pellet was resuspended by vortexing in 100µl of an ice cold solution of 50mM glucose, 10mM EDTA, 25mM Tris-Hcl (pH 8.0), and 4 mg/ml lysozyme. After sitting for 5 minutes at room temperature, 200 µl of fresh 0.2N NaOH and 1% SDS were added, and the tubes were inverted 2-3 times, then allowed to sit on ice for 5 minutes.

After 5 minutes on ice, 150 µl of cold potassium acetate (pH 4.8) were added, and the tubes were vortexed and allowed to sit on ice 5 minutes. The tubes were centrifuged 5 minutes at 4°, then the supernatant was transferred to a

fresh tube. To the new tube was added an equal volume of phenol/chloroform, and the solution was vortexed, then centrifuged for 2 minutes at room temperature.

The supernatant was again transferred to a fresh tube, and 2 volumes of EtOH were added. The tubes were vortexed and allowed to stand for 2 minutes. After centrifugation for 5 minutes at room temperature, the supernatant was removed, and the tube was inverted for drainage. 1 ml 70% cold EtOH was added, and the solution was centrifuged 2 minutes at room temperature. The supernatant was removed, and the pellet was dried in a vacuum dessicator. 50 µl TE (pH 8.0) were added, and the pellet was resuspended by vortexing.

Restriction enzyme analysis of the subclones. DNA samples from the minipreps were digested with EcoRI and SalI and their appropriate buffers, then were electrophoresed on a 0.7% agarose gel under 20-30 volts. The appropriate subcloned *revertant* DNA, *psn9* DNA, and sn^{cm} DNA were then subjected to restriction digests of EcoRI and AvaII (and appropriate buffers) and electrophoresed under similar conditions. These same DNA samples were also digested with BalI in a separate electrophoresis experiment.

Southern blotting. The EcoRI-AvaII and BalI digest gels were Southern blotted onto NYTRAN (Schleicher and Schuell protocol). The NYTRAN filter was soaked in 10 X SSPE, and the gel was soaked in 1.0M NaCl/0.5M NaOH twice for 15 minutes each. The gel was then neutralized in 1.5M NaCl/0.5M Tris (pH 7.4), 15 minutes. Transfer of DNA was by capillary method, overnight (Figure 18). The NYTRAN paper was then washed in 5 X SSPE for 5 minutes, then baked for 1 hour.

The NYTRAN filter was hybridized overnight to $^{32}P\pi 25.1$ DNA, at 37° in 50% formamide, 5 X SSC, 10% dextran-sulfate, 2 X Denhart's solution, 0.05M phosphate buffer (pH 6.8), and 100 µg/ml sonicated salmon sperm DNA (pre-hybridization solution). The filter was washed in 0.1% SDS, 10mM Tris-HCL (pH 8.0) at room temperature for 1 hour, then was allowed to

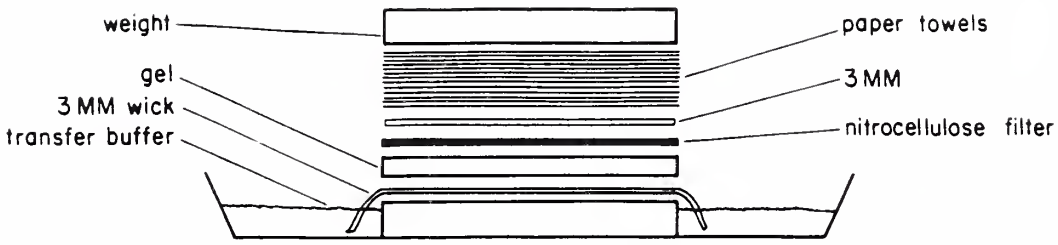


Figure 18--Capillary method used in Southern blotting (Maniatis et al., 1982).

dry and was exposed to X-ray film (autoradiogram).

Results

Cloning of genomic libraries. Of the 3 samples of *revertant* DNA obtained from the Hawley lab (*sn^{revs} 645-15*, *645-35*, and *645-60*), only *sn^{revs} 645-15* and *645-35* were successfully digested with EcoRI and were able to undergo ligation. *sn^{rev} 645-60* was absent from the gel run after the EcoRI digestion, and further work on this mutant was abandoned (Figure 19). A Southern blot of this genomic digestion using probe *psn9* indicated hybridization to DNA in the *645-35* and *645-15* lanes, but absence of any hybridization from the *635-60* sample (Figure 20).

Digestion of genomic DNA by EcoRI produces fragments of DNA that are less than 20 kb long. These are easily ligated to lambda DNA. The fragment that contains *singed* is contained within these pieces.

The 2 samples that were successfully digested and ligated were plated, and screening with *psn9* probe followed. Combining phage DNA with C600*Hfl*$^{+}$ cells allows the phage to infect the cells, which then amplify the phage DNA and lyse, continuing to grow as a plaque on the LB nutrient plates. Theoretically, each plaque has a different segment of genomic DNA. The NYTRAN filters lift the DNA directly from the plaques on the plates and allow the DNA to be hybridized to other single-stranded fragments.

Probe *psn9* is a 5.2 kilobase EcoRI fragment from the 5′ region of the wild type *singed* locus and was cloned by Roiha, O'Hare, and Rubin, and obtained from Rubin (Hawley, 1988). This probe allowed detection of the plaques that contained phage DNA with the *singed* locus. The appropriate plaques appeared as dark spots on autoradiograms (Figure 21), due to localization of the hybridized radioactive probe. These colonies were picked and replated a second and third time,

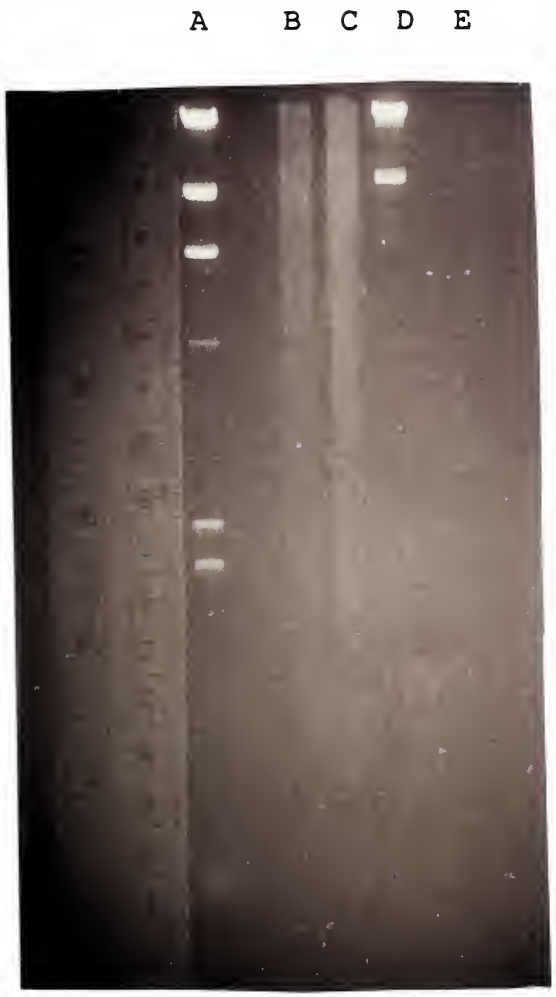


Figure 19--Agarose gel containing the EcoRI genomic digestions of samples *sn^{rev} 645-15*, *645-35*, and *645-60*. (A) λHindII size standard, (B) *sn^{rev} 645-15*, (C) *sn^{rev} 645-35*, (D) *λgt10*, (E) *sn^{rev} 645-60*.

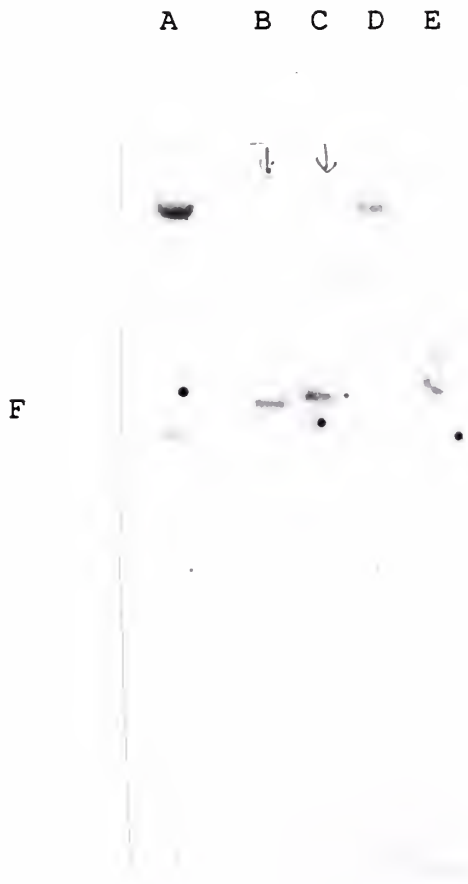


Figure 20--Southern blot of genomic EcoRI digest, probed with *psn9*. (A) λHindIII size standard. (B) *645-15* (C) *645-35* (D) λ*gt10* (E) *645-60* (F) Fragment of DNA in samples *645-15* and *645-35* that hybridized to *psn9*, absent in *645-60*.

until single colonies could be isolated from plates on which all colonies hybridized to probe.

Several attempts to screen the *645-35* library were unsuccessful beyond the secondary screening. The cloning of this *revertant* was eventually abandoned.

Subcloning of *revertant* DNA. *sn^{rev} 645-15* DNA was prepared for subcloning using the "minilysate" procedure to obtain large quantities of the desired DNA. A test gel of EcoRI-digested *revertant* DNA confirmed the presence of an approximately 5.2 kb band (Figure 22), the desired band length (Figure 23). An EcoRI and SalI digest of *revertant* DNA and plasmid *pT7-1* DNA again showed the presence of appropriate bands (Figure 24)--3.1 and 1.9 kb bands for the *revertant* DNA, and a 2.8 kb band for the plasmid (Figure 25).

The EcoRI/SalI digest gel was Southern blotted and probed with *psn9*. The 3.1, 1.9, and 2.8 kb bands hybridized (Figure 26), confirming the presence of the appropriate bands. Afterwards, the *revertant* DNA was ligated to the plasmid (Figure 27).

The subcloning of the *revertant* fragments into the plasmid helped to isolate the desired fragment within a vector which is easily taken up by competent cells and can be readily reproduced by these cells.

Transformation of cells. The transformation procedure requires cells to receive the plasmids containing the *revertant* DNA. When the plasmids are added to the competent cells, they are taken up by the cells and are reproduced en mass.

Minipreps. Once the cells were transformed with plasmids, they were plated, and single colonies were picked and cultured overnight. The alkaline lysis method was one way to isolate the plasmid DNA from the cell DNA. Once the plasmid DNA was isolated, an EcoRI/SalI digest was performed on the samples, and the samples showing the appropiate 2.8 (plasmid) and 1.9 kb (right side of the *sn* gene) bands on gel electrophoresis were identified (Figure 28). Samples

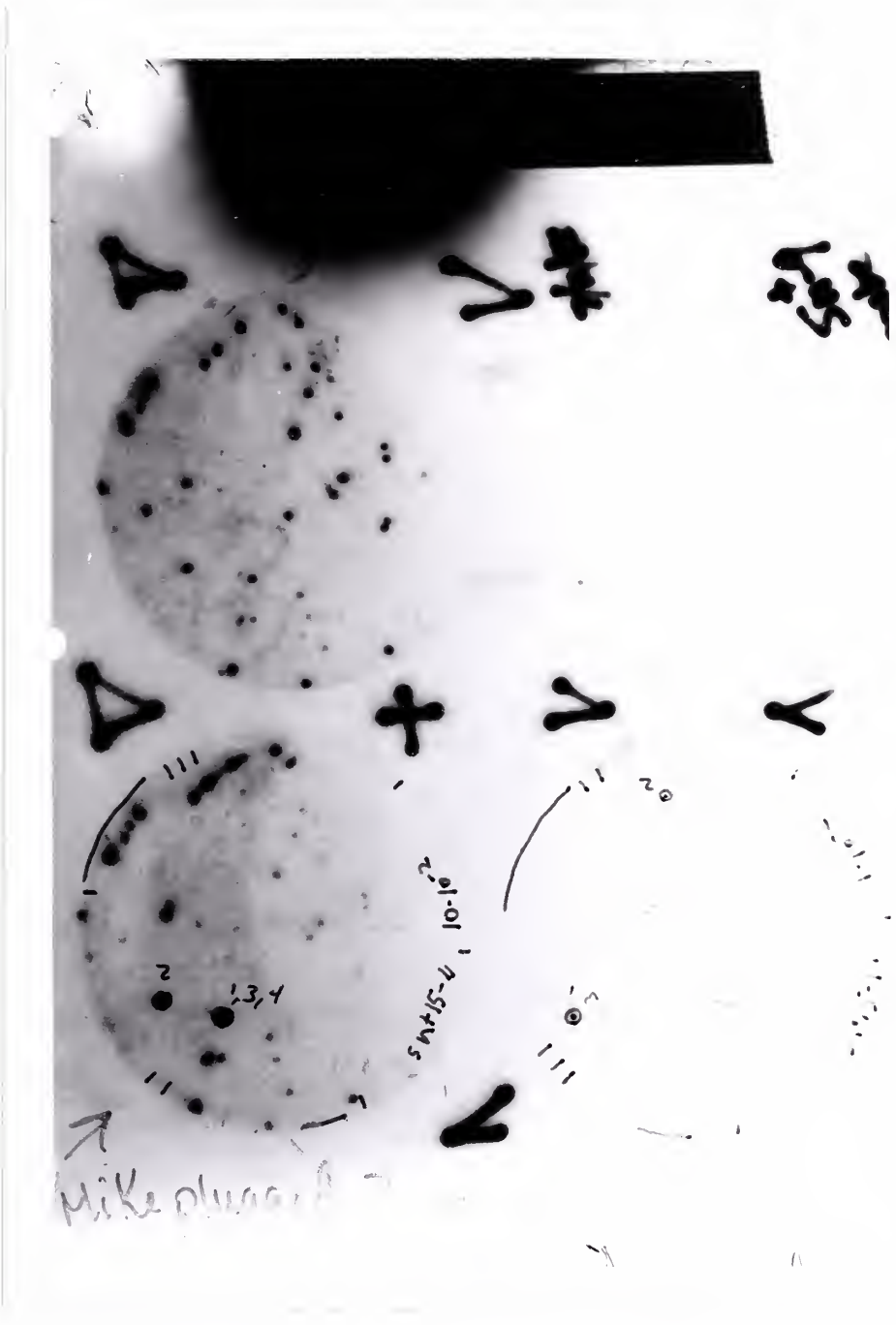


Figure 21--Example of autoradiogram of NYTRAN filters lifted off genomic library plates and hybridized to *psn9*--secondary screening of *645-15* library. Colonies that "lit up" were plugged and replated for a tertiary screening.

Figure 22--EcoRI digest of *sn*^{rev} *645-15*. (A) λHindIII size standard. (B) *645-15*. (C) 5.2 kb *sn* band.

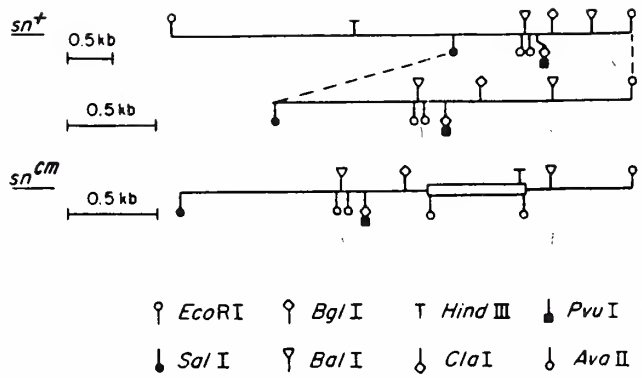


Figure 23--Restriction maps of the 5.2 kb *sn*$^{+}$ and 5.8 kb *sn*^{cm} loci. *P* element represented by thicker bar (Hawley et al., 1988).

Figure 24--EcoRI/SalI digest of *645-15* and *pT7-1*. (A) *645-15* and *pT7-1* samples. (B) λHindIII size standard. (C) 3.1 kb *sn* band. (D) 2.8 kb *pT7-1* band. (E) 1.9 kb *sn* band.

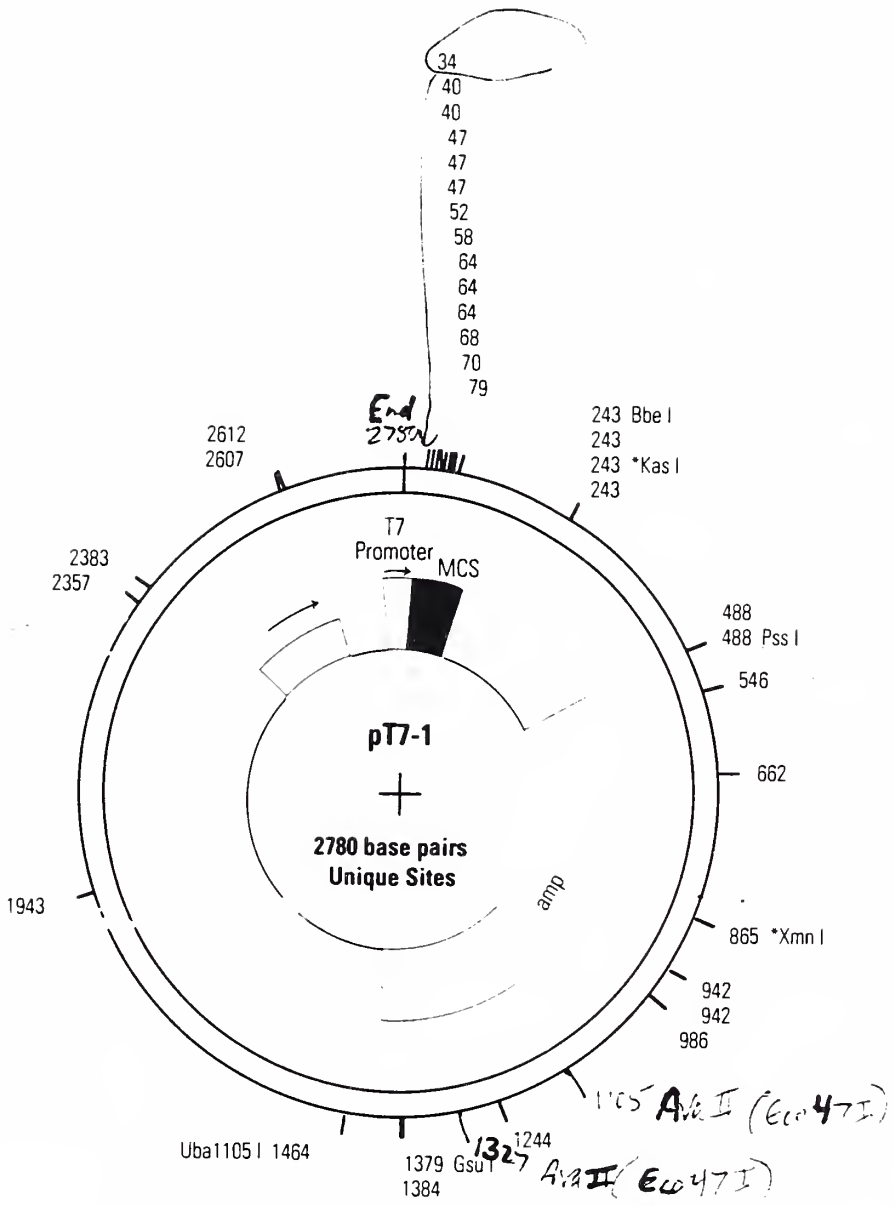


Figure 25--Restriction map of *pT7-1* plasmid (U.S. Biochemical).

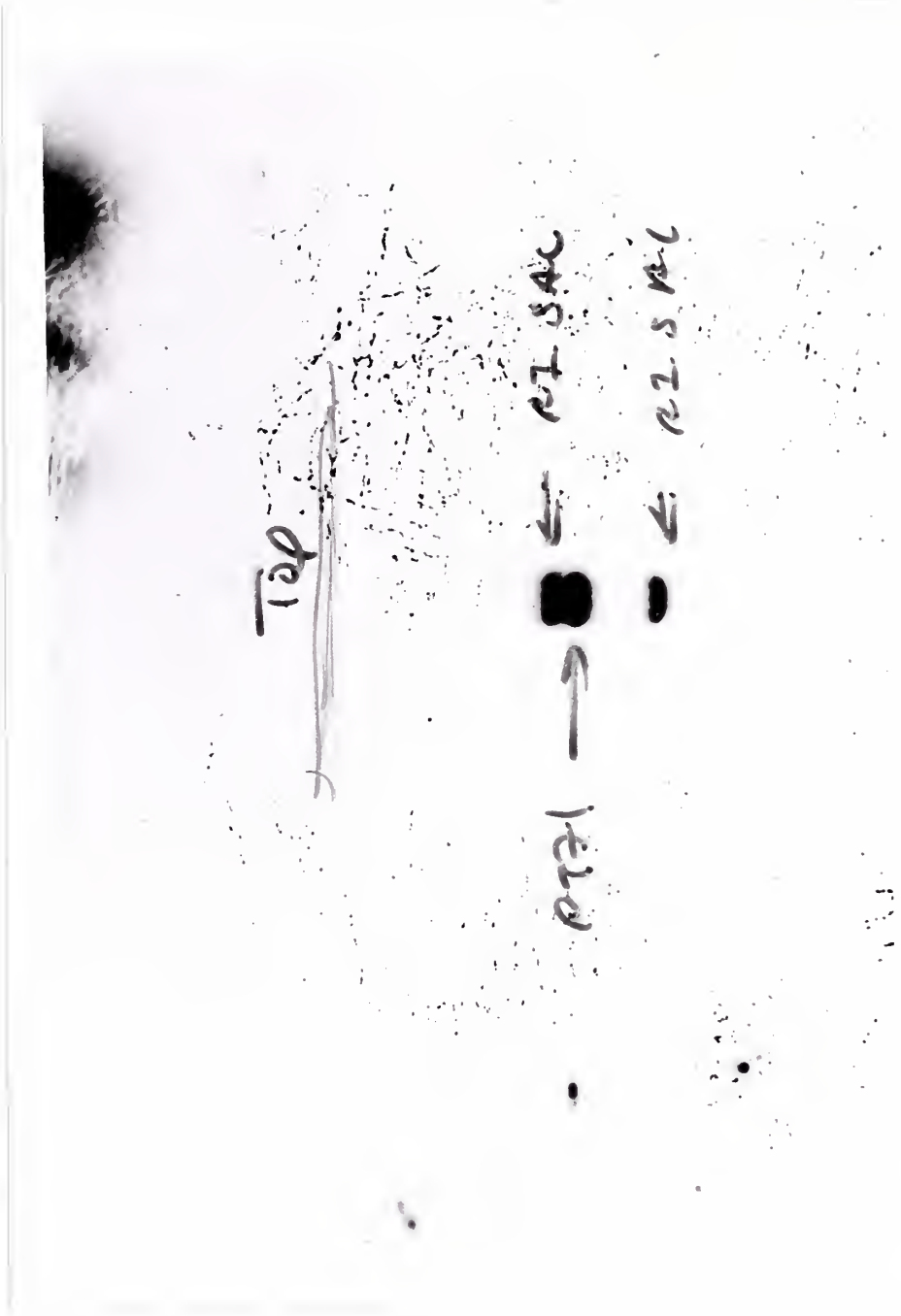


Figure 26--Southern blot of EcoRI/SalI digest of *645-15* and *pT7-1*, probed with *psn9*. (A) 3.1 kb *645-15* band. (B) 2.8 kb plasmid band (C) 1.9 kb *645-15* band.

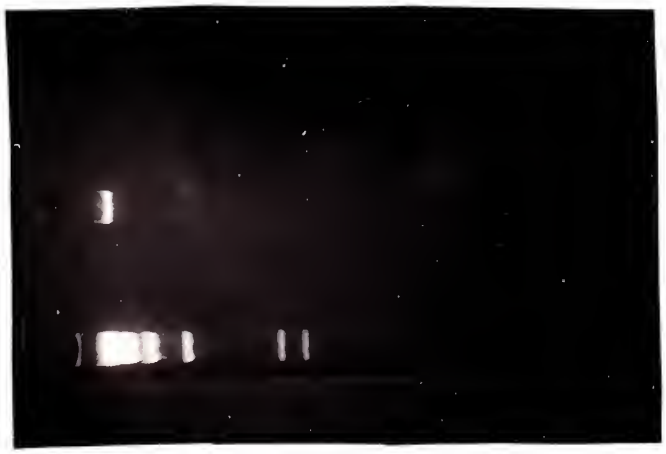


Figure 27--Ligation of *sn*^{rev} *645-15* to *pT7-1*. (A) Single band in lane containing ligated sample indicates successful ligation. (B) λHindII size standard.

containing the 2.8 kb plasmid band and the 3.1 kb *singed* band (left side of the locus) were not analyzed further. More DNA was prepared from one of the appropriate samples, and again, a test portion was electrophoresed after a similar enzyme digest to confirm isolation of desired fragments (Figure 29). This DNA was then used for several restriction enzyme digestions.

Restriction enzyme analysis. Restriction site maps of the *singed* locus and of the 628 bp *P* element were obtained from the Hawley lab, while a similar map of the *pT7-1* plasmid was obtained from U. S. Biochemical (Figures 24 and 26, respectively). An understanding of where certain enzymes cut the DNA within the *singed* locus, *P* element, and plasmid allowed for an estimation of the different fragment lengths that would be generated using certain enzymes.

After digestion, the DNA was loaded into an agarose gel, which provides a medium for electrophoresis. By running a current through the gel, the negatively charged DNA migrated toward the positive terminal, the shorter DNA fragments travelling at faster speeds.

To estimate the sizes of the bands in question, a lambda DNA size standard, cut with HindIII, is run concomitantly. The size standard always produces bands of DNA whose lengths are known (0.6 kb, 2.0 kb, 2.2 kb, 4.4 kb, 6.6 kb, 9.0 kb, and 23.1 kb). By logarithmically graphing the size of the lambda bands (kb) against distance migrated (cm), the sizes of the unknown DNA bands may be determined by referring to the generated graph.

DNA from the subcloned *revertant*, from a sn^{cm} sample, and from *psn9* (wild type) was subjected to an EcoRI/AvaII digest, then was electrophoresed on a 0.8% agarose gel. The result generated a 1.1 kb band in both the *revertant* and *psn9* lanes, and bands of 0.6, 0.55, and 0.46 kb in the sn^{cm} lane (Figure 30). The *singed-cm* DNA possesses AvaII sites at the ends of its *P* element insert in addition to AvaII and EcoRI sites found within the *singed* DNA. The *psn9* DNA has no *P*

Figure 28--EcoRI/SalI digest of *645-15* plasmid preps. (A) λHindIII size standard. (B) Examples of subclone with 3.1 kb *revertant* and 2.8 kb plasmid bands. (C) Example of subclone with 1.9 kb *revertant* and 2.8 kb plasmid bands. (D) 3.1 kb *revertant* band. (E) 2.8 kb plasmid band. (F) 1.9 kb *revertant* band.

Figure 29--EcoRI/SalI digest of plasmid preps amplified from positive subclone sample. All lanes have bands of 1.9 kb (*revertant* DNA) and 2.8 kb (plasmid DNA). (A) λHindIII size standard. (C) Likely 0.5 kb band of bacterial DNA insert.

element and consequently lacks the additional AvaII sites. Thus, 0.55 and 0.46 kb bands (totalling 1.1 kb) were produced in the *sn^{cm}* DNA whereas a single 1.1 kb band resulted in the wild type DNA. The 0.6 kb band seen in *singed-cm* is the *P* element.

The presence of a 1.1 kb band in the revertant's DNA indicates the absence of the additional AvaII sites found with the insertion of the 628 bp element. Moreover, the apparent excision has left a single band equal is size to that seen in the wild type, indicating that *P* element excision has neither left behind nor removed DNA. This resolution is accurate to within the distance between the AvaII sites and the actual ends of the *P* element.

The same 3 samples were subjected to another restriction enzyme digest using AvaII alone. Results from the gel (Figure 31) showed bands of 0.6 kb, 0.46 kb, and 2.1 kb in the *singed-cm* lane. Again, because of the addition of 2 AvaII sites from the *P* element, the smaller bands could be predicted. The larger 2.1 kb band included the 0.55 kb fragment between the end of the *P* element and the EcoRI site joined to ~ 1.5 kb of plasmid DNA between the same EcoRI site and the next AvaII site on the plasmid.

In contrast, a 2.6 kb band was present in the revertant's gel lane. This band includes the same 1.5 kb from the plasmid, as well as the 1.1 kb of subcloned *singed* locus, without the 0.6 kb *P* element and its AvaII sites.

A similar digest with BalI alone (Figure 32) demonstrated matching 0.75 kb bands in the wild type and *revertant* lanes, while showing a lone 1.35 kb band in the *sn^{cm}* DNA. The 0.75 kb band is expected to be the length between BalI sites in a *singed* locus without an insert, while the 1.35 kb band results from the addition of the insert.

Southern blot analysis. The Southern blot technique allows DNA that has been run on an agarose gel to be absorbed onto a filter which may then be hybridized to a radioactive DNA probe. In this fashion, DNA can be sized from a gel and

Figure 30--EcoRI/AvaII digest of *psn9*, sn^{cm}, and *645-15 revertant* samples. (A) λHindIII size standard. (B) sn^{cm} sample. (C) *645-15* sample. (D) *psn9* sample. (E) 2.1 kb fragment. (F) 1.8 kb fragment. (G) 1.5 kb fragment. (H) 1.1 kb fragment. (I) 0.6 kb *P* element. (J) 0.55 kb band. (K) 0.46 kb band.

Figure 31--AvaII digest of *sn^{cm}*, *645-15 revertant*, and *psn9*. (A) λHindIII size standard. (B) *645-15* sample. (C) *sn^{cm}* sample. (D) *psn9* sample. (E) 2.6 kb band. (F) 2.1 kb band. (G) 1.8 kb band. (H) 0.6 kb *P* element band. (I) 0.46 kb band.

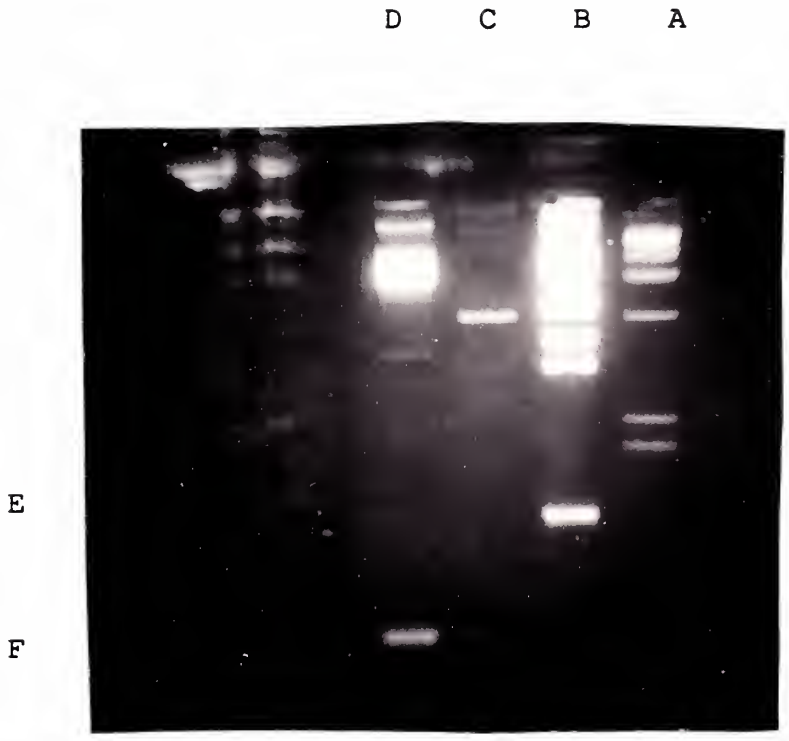


Figure 32--BalI digest of *sn^{cm}*, *645-15 revertant*, and *psn9* samples. (A) λHindIII size standard. (B) *sn^{cm}* sample. (C) *645-15* sample. (D) *psn9* sample. (E) 1.35 kb fragment. (F) 0.75 kb fragment.

then identified positively by hybridization.

The EcoRI/AvaII digest of *revertant*, wild type, and sn^{cm} DNA was Southern blotted and hybridized to $^{32}P\pi 25.1$ probe, which consists of plasmid DNA and a full length *P* element (Spradling and Rubin, 1982). The result indicated that the probe did not hybridize to the 1.1 kb band seen in the revertant's gel lane, implying the absence of any remaining *P* element in the locus (Figure 33).

A similar blot of the BalI digest using the same probe showed a similar absence of hybridization to the 0.75 kb bands in either the wild type or *revertant* lanes (Figure 34). However, hybridization occurred with the 1.35 kb band in the *singed-cm* lane.

Discussion

Interpretation of restriction enzyme digests. The samples of DNA interpreted to be successful subclones of the 5.2 kilobase *singed revertant* DNA were chosen from miniprep samples digested with EcoRI/SalI and run on agarose gel. These samples had bands that measured approximately 1.9 and 3.1 kb, lengths that correlate to those expected according to enzyme maps of the *singed* locus (Figure 23).

Additionally, in the miniprep samples selected there is another band of DNA measuring 0.5 kb. This is probably a SalI insert of bacterial DNA left over from the transformation of plasmids into cells.

In the EcoRI/AvaII digest of *psn9*, sn^{cm}, and sn^{rev} *645-15*, the 1.1 kb fragment in the revertant's lane is identical to that seen in the wild type. The AvaII sites within the *P* element appear to be deleted without visible loss of genomic DNA. The bands measuring 0.6, 0.55, and 0.46 kb in the sn^{cm} lane correlate to the same region of DNA as the 1.1 kb bands, with the addition of the 0.6 kb *P* element and its AvaII sites.

Furthermore, in the same digest, the 1.5 kb band shared

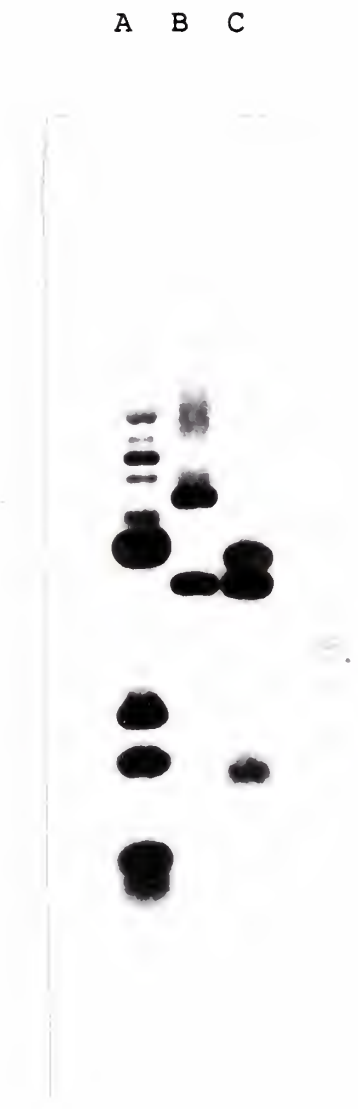


Figure 33--Southern blot of EcoRI/AvaII digest of *psn9*, *645-15*, and *sn*^{cm} DNA, probed with ^{32}Pπ25.1. Arrow indicates absence of hybridization of probe from the 1.1 kb band in the lane of *645-15* (B), and slight hybridization to same band in lane of *psn9* (A). (C) *sn*^{cm}.

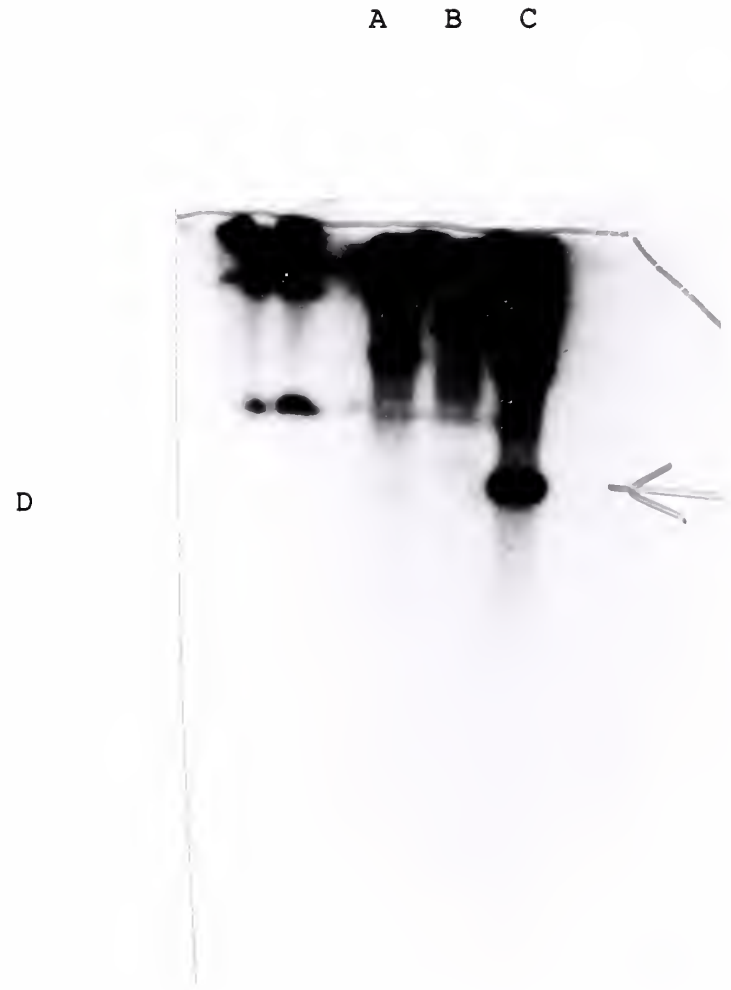


Figure 34--Southern blot of BalI digest of *psn9*, *645-15*, and *sn^{cm}* DNA, probed with ^{32}Pπ25.1. Arrow indicates hybridization of probe to 1.35 kb band in *sn^{cm}* (C), but absence of hybridization from wild type (A) or revertant (B), in area around their 0.75 kb bands (D).

by *sn*^{cm} and the *revertant* is plasmid DNA between the EcoRI site of the plasmid and a nearby AvaII site. The ~ 1.8 kb band seen in the *singed-cm* lane is also plasmid DNA between AvaII sites. This same 1.8 kb band is expected in the revertant's band pattern as well, but in its place is a 2.1 kb band. The extra ~ 300 base pairs are contributed by the previously discussed bacterial DNA.

In the AvaII digest of the same three samples, there is a 2.6 kb band in the revertant's lane. This consists of 1.1 kb of genomic DNA plus the adjacent 1.5 kb of plasmid DNA. The 2.1 kb band also seen is the same fragment seen on the EcoRI/AvaII digestion, including the additional bacterial DNA insert.

The 2.6 kb AvaII band is absent from the *singed-cm* lane. Instead there are three bands: 0.6, 0.46, and 2.1 kb. The 0.6 kb band is the inserted *P* element, and the 0.46 kb band is DNA between an AvaII site on the *P* element and an AvaII site on the genomic DNA. The 2.1 kb fragment is produced from the 0.55 kb of DNA between the other AvaII site on the *P* element and the EcoRI site on the genomic DNA, in addition to the 1.5 kb of adjacent plasmid DNA. The 1.8 kb band also seen corresponds to the same fragment of DNA on the EcoRI/AvaII digest. Thus, the AvaII digest adds creedence to the deduction that *P* element is absent from the *revertant*, and excision is essentially precise.

As further evidence, the BalI digest of wild type, *revertant*, and *singed-cm* DNAs indicates a 0.75 kb band shared by *psn9* and the *revertant*. This is the fragment length expected between BalI sites on the *singed* locus in the absence of a *P* element insertion. The 1.35 kb fragment in the *sn*^{cm} DNA reflects the addition of the *P* element. Thus, the BalI digestion provides a third means to indicate a precise excisional event in the *revertant*.

Interpretation of the Southern blots. The blot of the EcoRI/AvaII digest, which was hybridized to π25.1 probe (a plasmid with a full length *P* element), indicated that

hybridization occurred at the 0.6 kb fragment (the *P* element insertion) of *singed-cm*. Hybridization also occurred at the plasmid DNA of *singed-cm* and *revertant*, but there was no hybridization seen at the 1.1 kb band of the *revertant*. The absence of hybridization at the latter strongly supports the conclusion that *P* element is completely absent from this portion of *singed* DNA in the *revertant*.

A slight hybridization of the probe to the 1.1 kb band of *psn9* is probably a reflection of a greater concentration of DNA in this sample (and hence greater probability of hybridization to any single-stranded DNA). To eliminate such a problem, perhaps hybridization conditions could have been made more stringent.

The Southern blot of the BalI digest using the same π25.1 probe indicated that hybridization occurred at the 1.35 kb fragment in the *singed-cm* lane. Hybridization was absent from the 0.75 kb bands in the gel lanes of both wild type and *revertant* DNA. Once again, the lack of hybridization to the 0.75 kb bands strongly supports the conclusion that the *P* element has completely excised from the *revertant* fly.

Conclusions. Excision of the *P* element from the *singed* locus of the *revertant* flies was documented by EcoRI digests of sn^{cm}, sn^{rev}, and sn^{+} DNA which were Southern blotted and probed with *psn9* (Hawley, 1988). This initial blot suggested that excision was precise (Figure 35).

The EcoRI/AvaII digests of the same genomic DNAs indicate that the 1.1 kb band seen in the *revertant* DNA, and matching that of the wild type DNA, could not have been generated if either one or both of the AvaII sites from the *P* element had remained behind after an imprecise excision. Since the AvaII sites on the *P* element are approximately 20 base pairs away from the actual ends of the element, excision had to encompass almost the entire *P* element, leaving behind a maximum of 40 bp. The fate of these 40 bases can only be understood by other methods. DNA sequencing would be the method of choice if one were to examine excision down to a

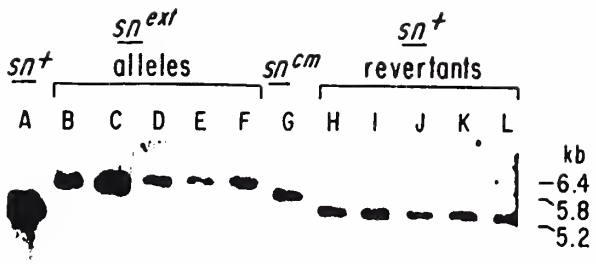


Figure 35--Southern blot analysis of *sn*^{ext} and *sn*$^{+}$ derivatives of *sn*^{cm} DNA. DNA was digested with EcoRI and probed with *psn9* (Hawley et al., 1988).

single base pair, and it would unequivocally answer the question of precise versus imprecise excision from the locus.

The failure of probe π25.1 to hybridize to either the 1.1 kb band in the Southern blot of the EcoRI/AvaII digest or the 0.75 kb band in the blot of the BalI digest reinforces the conclusion that no *P* element DNA was left behind during excision.

Similarly, if the *P* element had removed DNA from the *singed* locus during an imprecise excision, the band lengths seen in restriction enzyme digests of the *revertant* DNA would not have matched those seen in similar wild type digests. However, relevant bands from both *revertant* and wild type samples lined up precisely in the EcoRI/AvaII digest and the BalI digest. The smaller size (0.75 kb) fragment generated by the BalI digest showed that even subtle size differences appeared to be absent.

Thus, excision of the *P* element from the *singed* locus in *revertant* flies is essentially precise. There is no evidence in support of either an imprecise excision leaving *P* element DNA behind, or an imprecise excision removing DNA from the *singed* locus.

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